Matrix metalloproteinase-2 (MMP2) and myocardial dysfunction associated with urgent cardiac surgery

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ABSTRACT

Background: Current management of patients presenting with acute coronary syndrome (ACS) includes aggressive and expeditious revascularisation, including surgical revascularisation. However, early surgery following ACS is associated with high mortality, as subsequent global ischaemia induced during surgery is imposed on infarcted myocardium. Emerging evidence suggests that matrix metalloproteinases (MMPs), especially MMP2, may have an important role in the acute myocardial dysfunction seen after global ischaemia-reperfusion injury, by targeting intracellular functional and structural proteins.

Aims: To investigate whether MMP2 has a causative role in heart dysfunction when previously infarcted hearts were subjected to further global ischaemia-reperfusion, as occurs in cardiac surgery, with and without cardioplegic protection.

Methods and Results: MI was surgically induced in male Wistar rats, (250-350 g body weight) by in vivo ligation of the left anterior descending artery. The animals were recovered for 7 days prior to excision of hearts and isolated Langendorff perfusion, followed by induction of further global ischaemia and reperfusion. The recovery of mechanical function (left ventricular developed pressure: LVDP) of the heart was assessed during reperfusion. MMP2 activity was also measured during the early reperfusion phase in the heart tissues. Infarcted hearts had less capacity to recover function after an additional period of global ischaemia, which was associated with higher MMP2 activity in the infarcted hearts compared to normal hearts. Inhibition of MMP2 improved recovery of function. When an MMP inhibitor was used as an adjunct to St Thomas' Hospital cardioplegia, there was a trend towards improved recovery if the inhibitor was present before, during and after ischaemia.

Conclusion: MMP2 has a role in causing cardiac dysfunction when infarcted hearts were subjected to further global ischaemia-reperfusion. Inhibition of MMP2 resulted in improved recovery of the function of the hearts during reperfusion. With cardioplegia, MMP2 inhibition before, during and after ischaemia was crucial to improve cardioprotection during early reperfusion.

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MMP2 and myocardial infarction: can MMP2 inhibition prevent 'double-dip' dysfunction from elective (surgical) ischaemia-reperfusion? E S Teh and D J Chambers. *Circulation* 2012; 126: A12184.

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The influence of acute matrix metalloproteinase activity on myocardial dysfunction associated with urgent cardiac surgery: cardioprotective effects of inhibition. E S Teh and D J Chambers. *Journal of Interactive Cardiovascular and Thoracic Surgery* 2012; 15: S73

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The influence of acute matrix metalloproteinase activity on myocardial dysfunction associated with urgent cardiac surgery: cardioprotective effects of inhibition. E S Teh and D J Chambers. *The Journal of Heart Disease* 2012; 9: 101.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF COMMON ABBREVIATIONS

ACS	Acute coronary syndrome
AHA	American Heart Association
ANGII	Angiotensin II
ANOVA	Analysis of variance
APMA	p-aminophenylmercuric acetate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSU	Biological services unit
CABG	Coronary artery bypass graft
CF	Coronary flow
CGRP	Calcitonin gene-related peptide
CPB	Cardiopulmonary bypass
DTT	Dithiothreitol
EACTS	European Association for Cardiothoracic Surgery
ECG	Electrocardiogram
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMMPRIN	Extracellular matrix metalloproteinase inducer protein
ESC	European Society of Cardiology
ET	Endothelin
ETT	Endotracheal tube
GRACE	Global Registry of Acute Coronary Events
HR	Heart rate
HRP	Horseradish peroxidase
IHD	Ischaemic heart disease
IL-1β	Interleukin 1β
JAK-STAT	Janus kinase-signal transducers and activators of transcription
KO	Knockout
LAD	Left anterior descending artery
LMS	Left main stem
LV	Left ventricle
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
LVSP	Left ventricular systolic pressure
LVSWI	LV stroke work index
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NCX	Sodium-calcium exchanger

NF-κB	Nuclear factor κB
NSTEMI	Non ST-elevation MI
OD	Optical density
PAI-1	Plasminogen activator inhibitor 1
PCI	Percutaneous coronary intervention
PV	Pressure volume
PVDF	Polyvinylidene difluoride
RCT	Randomised controlled trials
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SMC	Smooth muscle cell
STEMI	ST-elevation MI
TBST	Tris buffer saline Tween
TEMED	Tetramethylethylenediamine
TF	Tissue factor
TIMP	Tissue inhibitor of MMP
TNF	Tumour necrosis factor
TTC	Triphenyl tetrazolium chloride
WT	Wild type

1 INTRODUCTION

1.1 Ischaemic heart disease (IHD)

In the UK, ischaemic heart disease (IHD) is very common. More than 1.4 million people suffer from angina, with 275,000 having a heart attack every year [1, 2]. It remains the number one killer in Western society. In 2010, one in five men and one in ten women died from deaths due to heart disease [3]. There were about 495,000 episodes of inpatient hospital admission due to ischaemic heart disease in 2011 [3]. Hence, it continues to impose a significant health and economic burden in this country.

1.2 Pathophysiology of ischaemic heart disease

IHD or myocardial ischaemia, occurs when perfusion or blood flow to the myocardium is inadequate to meet its metabolic demand. This is due to narrowing or obstruction of the coronary arteries from atheriosclerosis, thrombosis, embolism or spasm. It is more common in men, and the risk of IHD is increased in smokers, people with diabetes mellitus, hypertension, hypercholesterolaemia and family history of IHD. It can present as a chronic disease with chest pain or angina on exertion. However, it can also present acutely with sudden onset of chest pain, chest pain at rest or rapidly worsening chest pain. The acute presentation is now known as acute coronary syndrome (ACS), which includes the classical full-thickness or transmural myocardial infarction (MI).

Atheriosclerosis is by far the commonest cause of coronary artery disease [4]. Risk factors mentioned above result in damage to the endothelium, which in turn initiates a cascade of events leading to the formation of atheroma within the endothelium. Atheroma is classically composed of a fibrous cap with a lipid rich core. With endothelial damage, there is upregulation of adhesion molecules on the endothelial surface, causing transmigration of blood leucocytes such as mononuclear lymphocytes and T-cell phagocytes into the intima of the arterial wall [5]. As a consequence, smooth muscle cells (SMC) migrate from the tunica media into the intima layer. Once in the intima, SMCs continue to proliferate and secrete matrix metalloproteinases (MMPs) in response to its

environment. MMPs, in turn, modulate vascular cells causing activation, proliferation, migration, cell death, geometric remodelling and destruction of the extracellular matrix (ECM) of the arteries or myocardium. These changes will further propagate the inflammatory process resulting in a vicious cycle, with further development of the atheromatous plaques and remodelling of the arteries.

ACS arises due to acute disruption to the atheromatous plaques [5, 6]. There could be a through and through rupture of the fibrous cap (which is often fatal), superficial erosion of the cap, intraplaque haemorrhage or erosion of a calcified nodule (Fig 1.1).

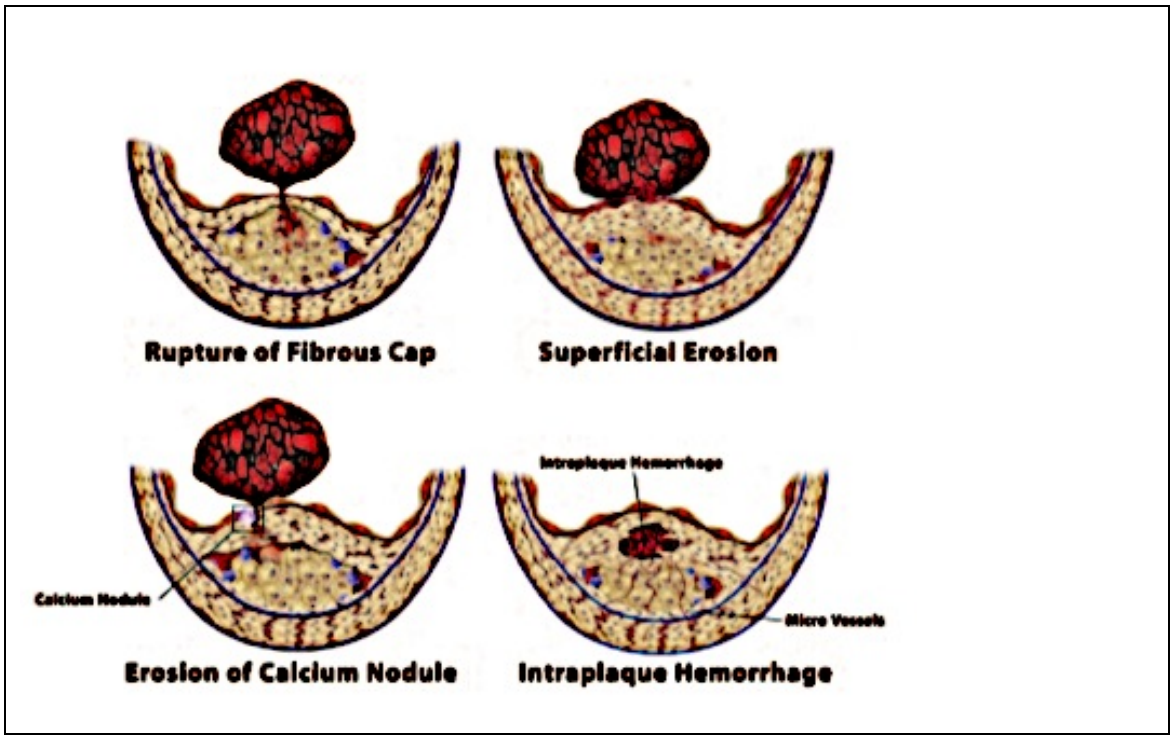


Figure 1.1 Microanatomy of coronary arterial thrombosis and acute occlusion. [5]

Disruption of atheromatous plaque exposes the collagen in the plaque ECM and activates platelets, whereas tissue factor (TF) secreted by the SMCs and macrophages activates the coagulation cascade. Besides, circulating plasminogen activator inhibitor-1 (PAI-1) in the blood comes in contact with the disrupted plaques, which also serves to increase the general thrombogenicity of the blood. As a result, thrombosis occurs and causes intraluminal obstruction of blood flow to the myocardium, resulting in the clinical symptoms experienced by patients.

The infarcted myocardium undergoes coagulative necrosis, with inflammatory changes such as neutrophil infiltration [7], followed by necrotic myocyte resorption, infiltration of fibroblasts and scar tissue formation and endothelial cell activation and neovascularisation. There are also complex structural and architectural changes as the myocardium heals and remodels [8], with thinning and dilation of the infarct zones and compensatory hypertrophy of the non-infarct zones. Depending on the severity, duration and location of the infarct, there are several possible complications as a consequence of myocardial infarction. These are cardiogenic shock, ventricular dysfunction, arrhythmias, myocardial ruptures (such as septal or free wall rupture), infarct extension and expansion. Myocardial infarction, with the consequent remodelling of the LV, could also ultimately lead to chronic heart failure in the long term.

Over the years, as a result of ongoing laboratory research, with clinical correlation, our understanding of the anatomy and biology of coronary arteriosclerosis have evolved. Inflammation is now known to be a central pathological process in all stages of atherogenesis [5]. Any important mediators of inflammation could be potential targets to reduce the risk of plaque rupture and limit the damage caused by the disease. Considering its likely role in plaque destabilisation and matrix remodelling, MMPs have emerged as proteins of interest in coronary artery disease.

1.3 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a ubiquitous group of zinc-containing endopeptidases involved in degradation of ECM, and mediating a variety of physiological functions such as embryogenesis, growth, angiogenesis and wound healing [9-12]. In addition, MMPs are involved in pathological processes, causing architectural extracellular matrix changes by participating in inflammation and carcinogenesis, vascular remodelling, advancing or destabilising atherosclerotic plaques, as well as inducing myocyte hypertrophy, apoptosis and interstitial fibrosis [11-15]. The parallel expansion in the understanding of the biology of the ECM (which is a dynamic structure rather than just a static structural scaffold) and the discovery of a vast and diverse array of non-ECM MMP substrates has gradually shifted the conventional

view of MMPs as destructive enzymes to important regulators in various cell-signalling processes [14].

1.3.1 Classification of MMPs

The classification of MMPs can be somewhat confusing. MMPs can be broadly divided into 4 different groups according to the substrate it acts on, or they can be classified numerically [12]; thus collagenases include MMP1 and MMP8, gelatinases include MMP2 and MMP9, stromelysins consist of MMP3, MMP10 and MMP11, and others such as MMP7, MMP12 and MT-MMP.

The protein structure of the different groups of MMPs is shown in Fig 1.2. The pro-domain, or NH_2-terminal domain, consists of about 80 amino acids and is made up of the signalling sequence and pro-peptide. The signalling sequence allows for secretion of the enzyme into the endoplasmic reticulum and transport out of the cell, whereas the pro-peptide sequence contains the cysteine switch. The cysteine switch plays a key role in activation of MMPs; this will be described in more detail in subsequent sections. Next is the catalytic domain, which is made up of about 170 amino acids, and also contains the zinc-binding motif. The cysteine switch in the pro-peptide domain and the zinc in the catalytic domain co-ordinate to maintain the enzyme in an inactive state. This NH_2-terminal domain and the zinc-binding motif form the common structural signature of MMPs. A flexible linker/hinge region joins the structure to the hemopexin domain. The hinge region is of different lengths in different MMPs, and is also absent in some MMPs such as MMP7, MMP23 and MMP26. The hemopexin domain, about 200 amino acids long, confers substrate specificity to MMPs by co-ordination of protein-protein interaction [11-13]. The hemopexin domain is linked to a C-terminal tail.

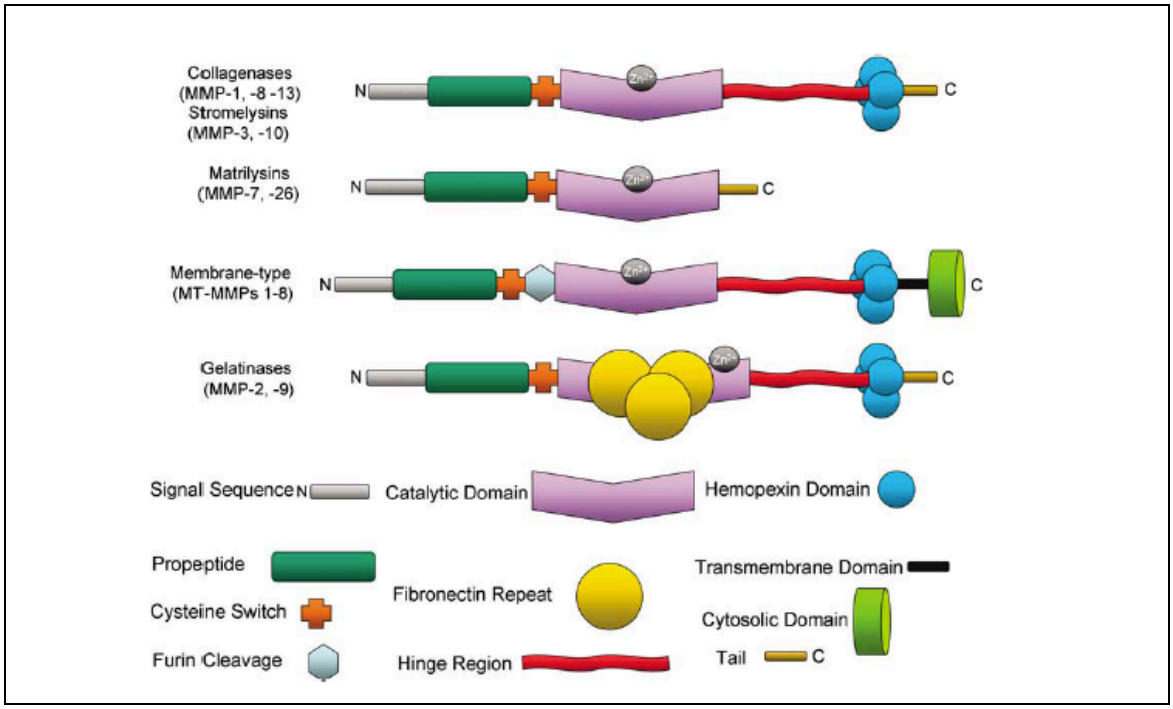


Figure 1.2 The structure of MMP.

MMPs consist of a pro-domain with cysteine switch, catalytic domain, joined by a linker/hinge region to the hemopexin domain. [13]

In tissues, MMPs are involved in degradation and removal of extracellular matrix. As the extracellular matrix is a dynamic structure, any alteration to its structure will alter cell-matrix and cell-cell interaction. There are abundant growth factors and bioactive molecules such as chemokines, cytokines and growth factors that are bound to the ECM. By altering the structure of the ECM, MMPs are therefore involved in the regulation, release and activation of these molecules. Hence, MMPs are important participants in the regulatory pathways in cellular proteolysis [11].

1.3.2 Regulation of MMPs

MMPs are tightly regulated at various levels due to their potential hazardous proteolytic activities. The earliest control of their activity is mediated via the modulation of their gene expression. The gene expression of MMPs can be stimulated by various factors. Up-regulation of MMP gene could be effected by soluble mediators such as growth factors and cytokines, cell-cell interaction, cell-matrix interaction and signaling pathways [16]. Bioactive molecules such as angiotensin II (ANGII), endothelin (ET) and

cytokines, typically tumour necrosis factor (TNF) and interleukin 1β (IL-1β), activate various signaling pathways such as Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, protein kinase-C and mitogen-activated protein kinase (MAPK) pathways, which in turn increases gene expression of various MMPs [12]. Mechanical stimuli such as changes in the cyclical myocardial strain pattern due to myocardial ischaemia, and manifested by the deformation in LV dimensions during the cardiac cycle, have also been shown to increase MMP expression [17, 18]. However, it is not entirely certain whether this is due to a direct action on transcription factors, or indirectly via oxidative stress and inflammatory markers in the myocardium that were also raised under these circumstances. Cell-cell and cell-matrix interaction, such as extracellular matrix metalloproteinase inducer protein (EMMPRIN), may also be an important factor in regulating the transcription of MMPs [19]. On the other hand, growth factors, via activation of SMAD pathways, could decrease the expression of MMPs.

MMPs are secreted as an inactive pro-enzyme into the extracellular environment and require stepwise activation (Fig 1.3). Initially, a conformational change leads to the disruption of the cysteine-zinc interaction, hence allowing a water molecule to bind to the catalytic zinc [10, 12, 13, 20]. There are a variety of triggers for this to occur. Conventionally, direct cleavage of the pro-domain (resulting in lower molecular weight MMPs), activates MMPs. Direct proteolysis of the pro-domain may be a result of an interaction between S-reactive agents, organomercurial compounds or reactive oxygen species and the conserved cysteine in the pro-domain. Alternatively, binding of chaotropic agents or detergents could also lead to the conformational change required to trigger the autocatalysis resulting in the stepwise degradation of the pro-domain [21].

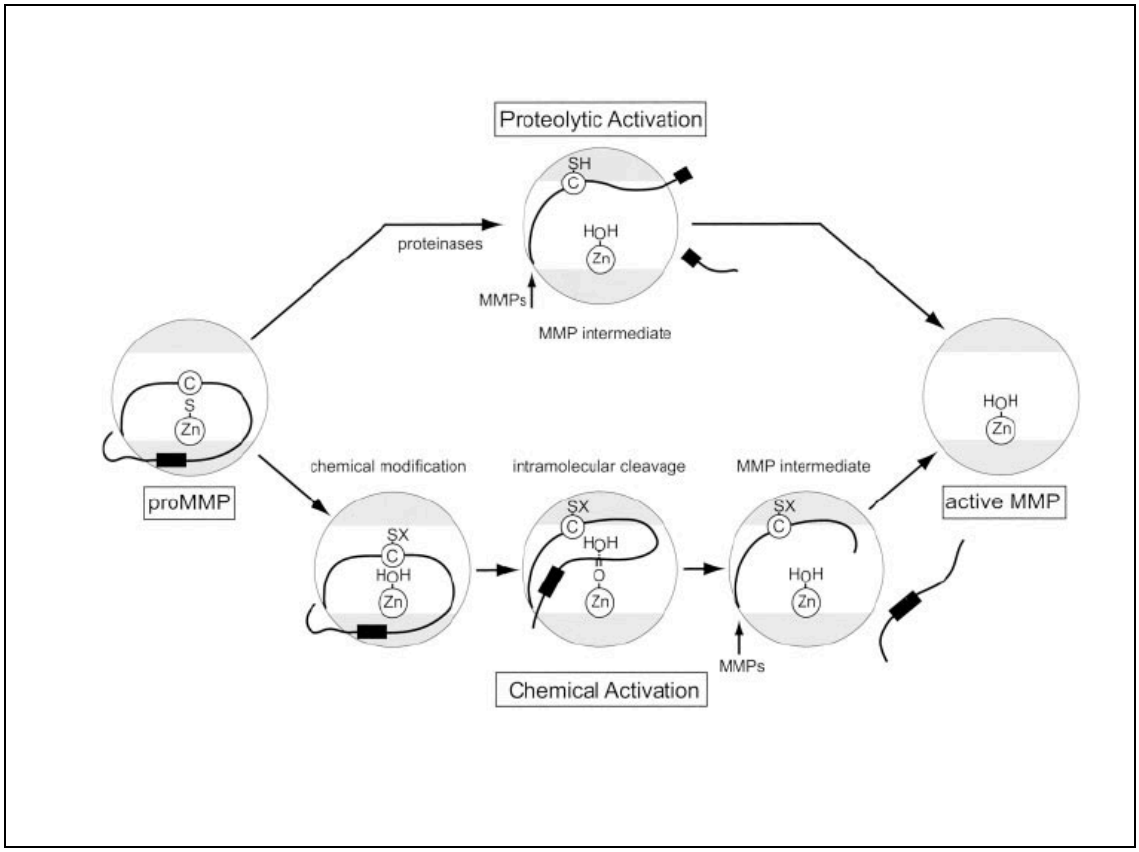


Figure 1.3 Schematic diagram of step-wise activation of pro-MMPs.

The top pathway represents the proteolytic pathway and the bottom pathway, the non-proteolytic pathway. The propeptide is represented by the black line with the black rectangle representing the bait region, where proteolysis occurs. C=cysteine switch, SH=sulfhydryl of cysteine, SX=cysteine switch. Chemical activation requires modification of SX, resulting in partial activation of MMP2, with intermolecular processing completing the activation process. [20].

More recently, it was discovered that direct cleavage of the pro-enzyme is not a necessary step to activate the MMPs. Allosteric reconformation or chemical modification of the pro-domain by thiol-modifying agents could also activate the MMPs [22]. Binding of macromolecules, or other proteins such as bone sialoproteins to pro-MMP2, could bring about the necessary allosteric reconformation [23]. Peroxynitrite and glutathione cause S-glutathiolation of cysteine, which will activate the MMPs [24-26]. There is recent evidence to suggest that phosphorylation and S-nitrosylation may also be important in regulating the activity of MMP [13, 27, 28]; however, the identities of the kinases and phosphatases that are involved are yet to be elucidated. These kinases and phosphatases may be novel targets involved in modulating the activity of MMP and its

impact on mechanical dysfunction of the myocardium. The other aspect of post-translational modification is localization or compartmentalization of MMPs so that it comes into contact with its potential substrate [21, 29]. This is facilitated by exosites, substrate-binding motifs outside the catalytic areas, and binding partners in the respective compartments. The exosites may also be important in the degradation of certain substrates and activation of intracellular signaling leading to events such as apoptosis.

Activated MMPs interact with their endogenous inhibitors; in tissues, tissue inhibitors of MMPs (TIMPs) and in circulating plasma, α-macroglobulin [12, 13]. They bind to MMPs in a 1:1 stoichiometric manner, and when bound will inactivate MMPs. The net activity of MMPs is a fine balance between activation and degradation. The tissue inhibitors of MMP (TIMP) and the complexes formed between MMPs and other proteins (proteoglycan core proteins and/or their glycosaminoglycan chains) are involved in the regulation of the balance between activation and degradation of MMPs.

1.3.3 Myocardial MMPs and Coronary Heart Disease

MMPs were first studied using zymography in patients undergoing heart transplantion. In the heart, MMPs are located within the endothelium, the subendothelial space of the endocardium and the interstitial space, usually in association with TIMPs [30]. The full spectrum of MMPs and TIMPs are found in the myocardium, with MMP2 mRNA level found to be specifically higher in heart tissues [30].

Following myocardial infarction, during remodelling, it was found that there is a type and region specific increase in MMPs (Fig 1.4) [31]. In the infarcted region, there was a significantly higher level of MMP activity, especially of MMP2, 13, 8 and MT1-MMP.

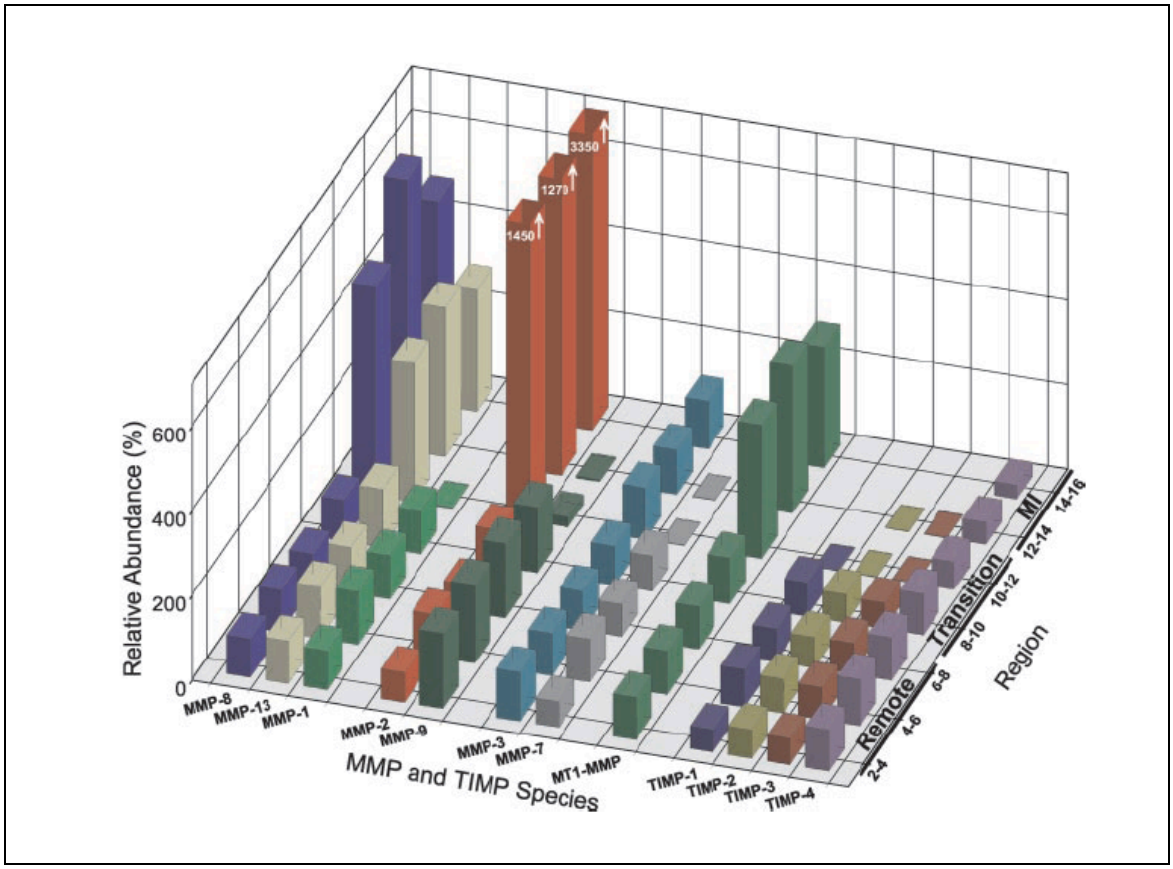


Figure 1.4 Histogram representing regional distribution of MMPs and TIMPs after MI.

There was a regional and type increase in MMP activity following MI. For example, in the MI region, levels of MMP2, MMP13, MMP8 and MT1-MMP were increased. [31].

Subsequent serial assessment showed that protein level and expression of MMP2 could increase as early as day one following infarction, peaked at day 2 and remained persistently high for about 5 weeks before gradually returning to baseline (Fig 1.5) [32, 33]. After 8 weeks post MI, there was a second surge of MMP2 protein level, but this second surge was not accompanied by parallel increase in its expression.

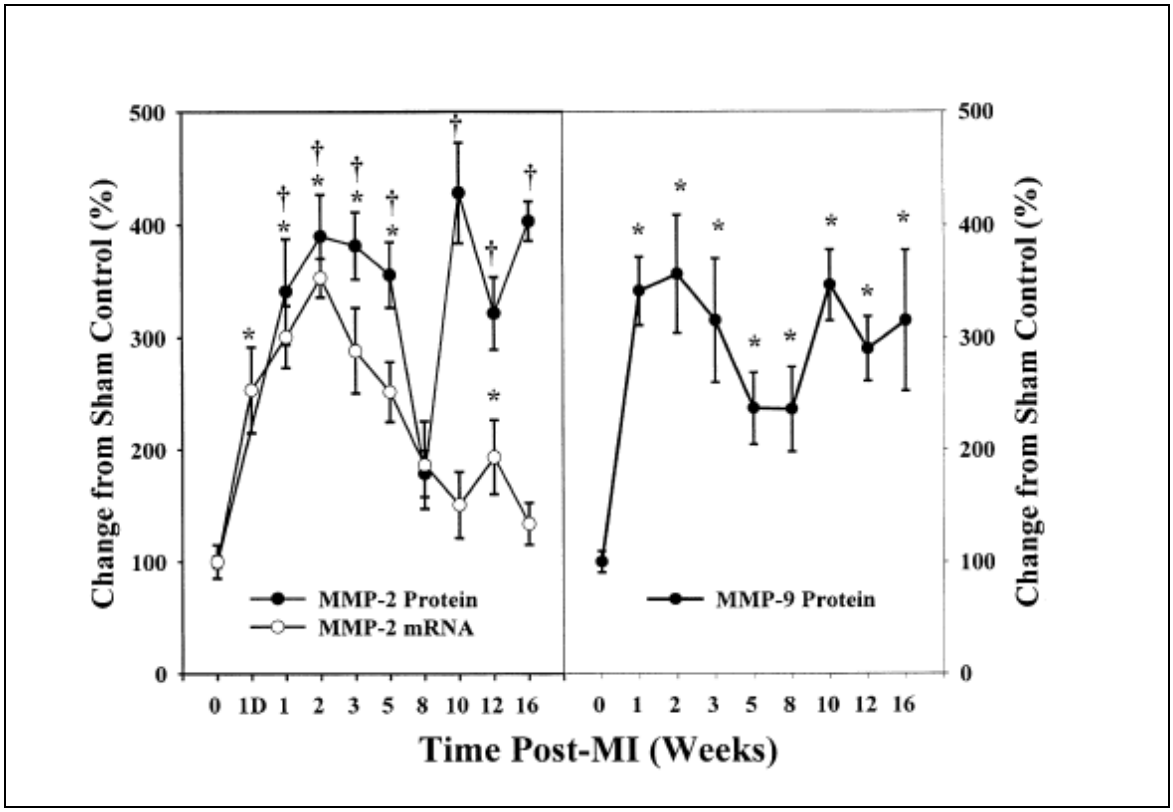


Figure 1.5 MMP2 mRNA and zymographic activity (left) and MMP9 zymographic activity (right) in infarcted (MI) rat hearts expressed as change (%) from sham-operated hearts [33].

MMP3 was also detected as early as day 2 post MI, peaked at day 4 and declined by day 14 [34] whilst MMP8 protein level increased later at day 14 post-ligation and remained elevated until 16 weeks post-ligation.

Although the action of MMPs on matrix remodeling takes hours or even longer, recent studies indicate that there may be a more acute response, suggesting a role in acute pathophysiology. In isolated Langendorff perfused heart preparations, MMP2 activity was shown to peak at 1 minute following reperfusion after a period of global ischaemia (Fig 1.6) [35]. The increase in activity was found to be proportional to the duration of ischaemia and inversely related to the recovery of the mechanical function of the heart (Fig 1.7).

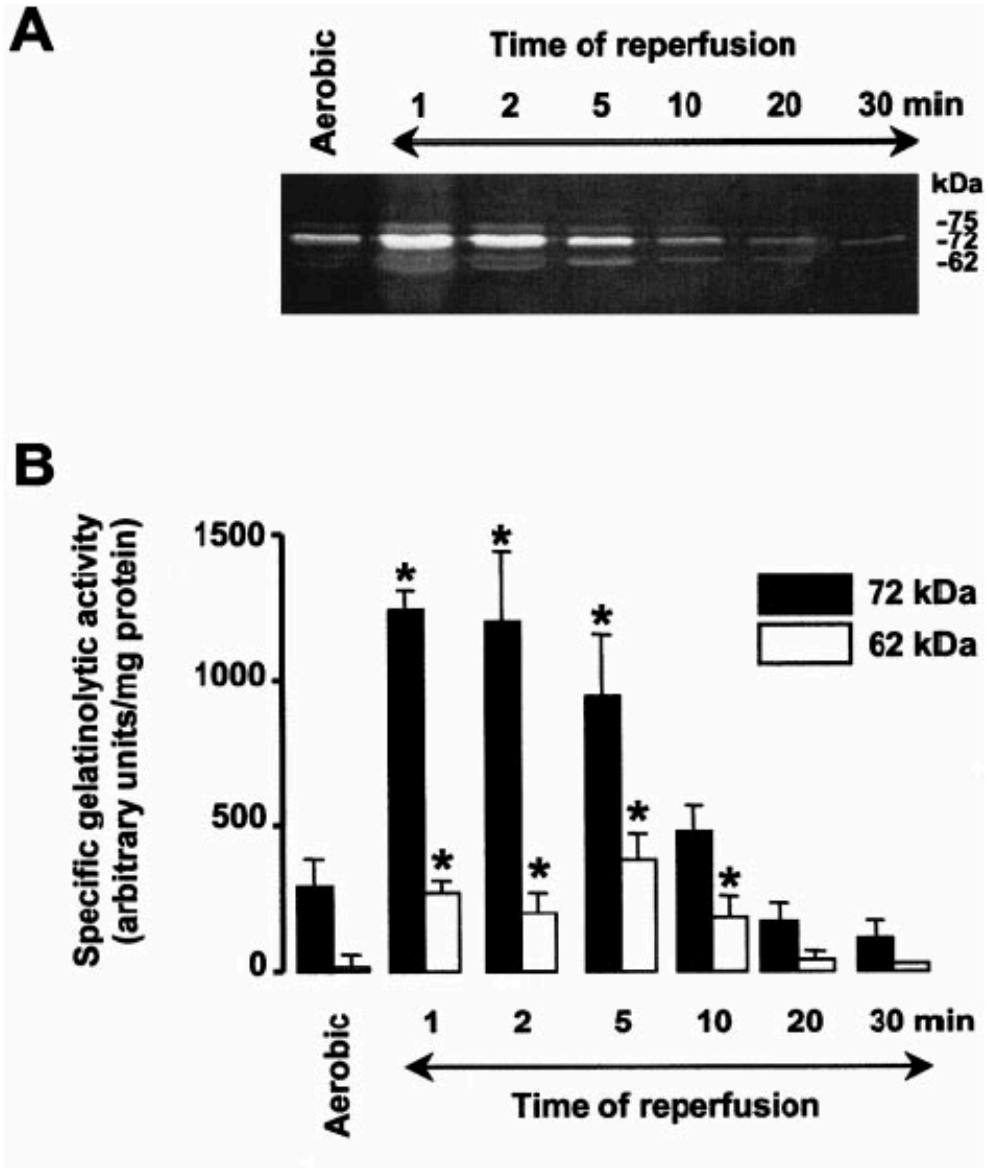


Figure 1.6 Gelatinolytic activities in coronary effluent of aerobically perfused rat hearts and during reperfusion after ischaemia.

A. Representative zymography showing gelatinolytic activities in coronary effluent during aerobic perfusion and at various reperfusion times after 20 min of global ischaemia. B. Densitometric analysis of specific gelatinolytic activities of pro-MMP2 (72 kDA) and MMP2 (62 kDA) in coronary effluent samples. [35]

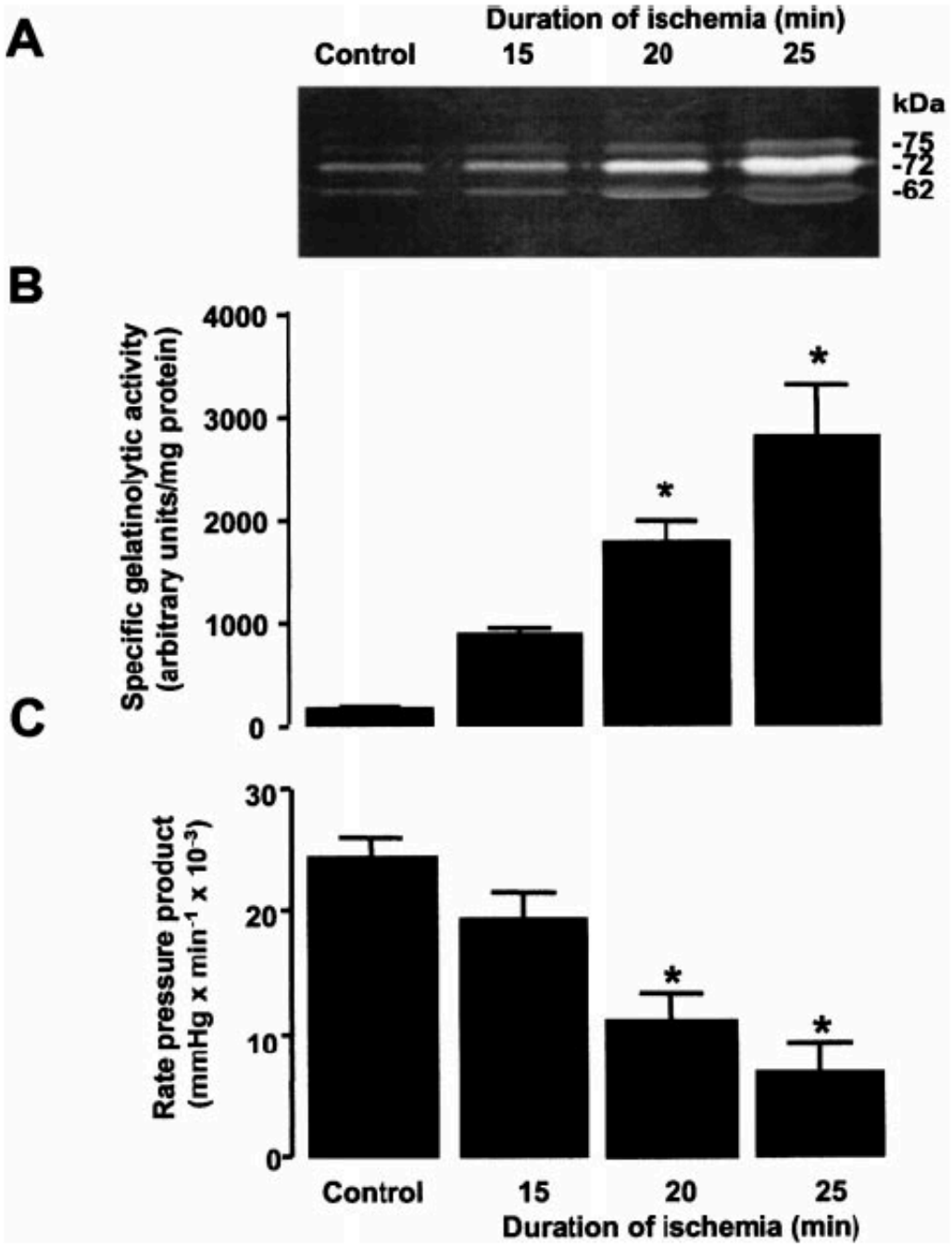


Figure 1.7 Relationship between duration of ischaemia, release of pro-MMP2 and recovery of function during reperfusion.

A. Representative zymogram of coronary effluent from control hearts and during the first min of reperfusion after 15, 20 and 25 min of global ischaemia. B. Densitometric analysis of gelatinolytic activities of pro-MMP2 in coronary effluents in experiments as in A. C. Recovery of mechanical function at 30 min of reperfusion. [35]

When MMP2 activity was inhibited with doxycycline and 1,10-phenanthroline, there was an associated improved recovery of the mechanical function of the heart (Fig 1.8). Conversely, when semi-purified MMP was added to the perfusate, the recovery of the heart was worse compared to controls.

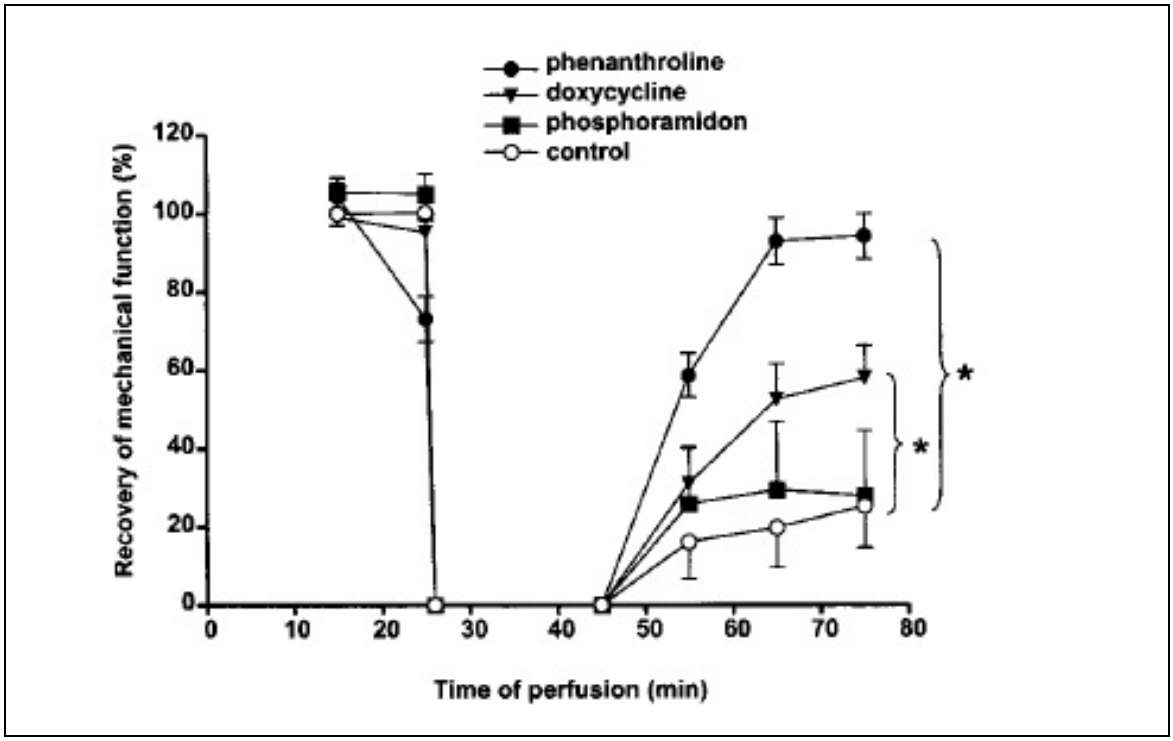


Figure 1.8 Effects of MMP inhibition on recovery of mechanical functions expressed as rate-pressure product (RPP). [35]

1,10-phenanthroline and doxycycline, but not phosphoramidon (which is an inhibitor of metalloproteinases but without any inhibitory effects on MMPs), caused a significant improvement in recovery of RPP compared to controls.

Subsequently, through various studies using immunohistochemistry and confocal microscopy, MMP2 was thought to mediate cardiac dysfunction in this setting of acute ischaemia-reperfusion by acting on various intracellular targets, such as troponin I, myosin light chain, α-actinin and titin, within the myocytes [36-38]. MMP2 was found to co-localise with various structural and functional proteins. When incubated with these proteins in vitro, MMP2 was able to proteolytically cleave these proteins, and the

proteolysis was inhibited with addition of MMP inhibitors such as doxycycline and 1,10-phenanthroline. The concept that ischaemia-reperfusion injury in myocytes is influenced by MMP2 via an independent mechanism to its actions on extracellular matrix is further reinforced in cell cultures and isolated myocyte studies [39, 40]. Valentin and colleagues showed, using cell cultures, that oxidative stress increases the activation of MMP2 via increased expression and activation of MT1-MMP and oxidative radicals produced via the xanthine/xanthine oxidase complex [40]. In an isolated myocyte study, Leon and associates showed that infusion of peroxynitrite decreased the contractility of the myocytes in a concentration-dependent manner, and that the effect was ameliorated by MMP inhibitors [39]. Besides intracellular target proteins, MMP2 could generate reactive oxygen species (ROS) or affect the integrity of the endothelium causing impaired myocyte contractility [41].

As mentioned earlier in this section, following an infarction, there was early and persistent increase in both protein and expression of MMP2. If a further ischaemia-reperfusion period is induced (as occurs during elective cardiac surgery), the associated injury may then activate a proteolytic cascade present within the myocardium resulting in synergistic detrimental effects on the function of the heart. Dixon and co-workers examined precisely this phenomenon, with particular attention to MT1-MMP in 2 groups of pigs [42]. In the first group, the animals were subjected to LAD occlusion for 60 min, followed by reperfusion for another 60 min (acute IR). In the second group, the animals first underwent surgical ligation of the circumflex artery, which is a different branch of the left coronary artery supplying a different territory of myocardium compared to the LAD. After 3 weeks, the second group of animals then underwent the exact same occlusion of LAD and reperfusion as the other group (acute on chronic IR). Haemodynamic measurements showed that at baseline (i.e prior to occlusion of LAD) and during ischaemia, the regional stroke work in group 2 (acute on chronic IR) was significantly worse compared to group 1 (acute IR) (Fig 1.9A). The activity of MT1-MMP at baseline was also higher both at the circumflex territory (infarcted myocardium) as well as at the LAD territory (normal myocardium) in group 2 (Fig 1.9B).

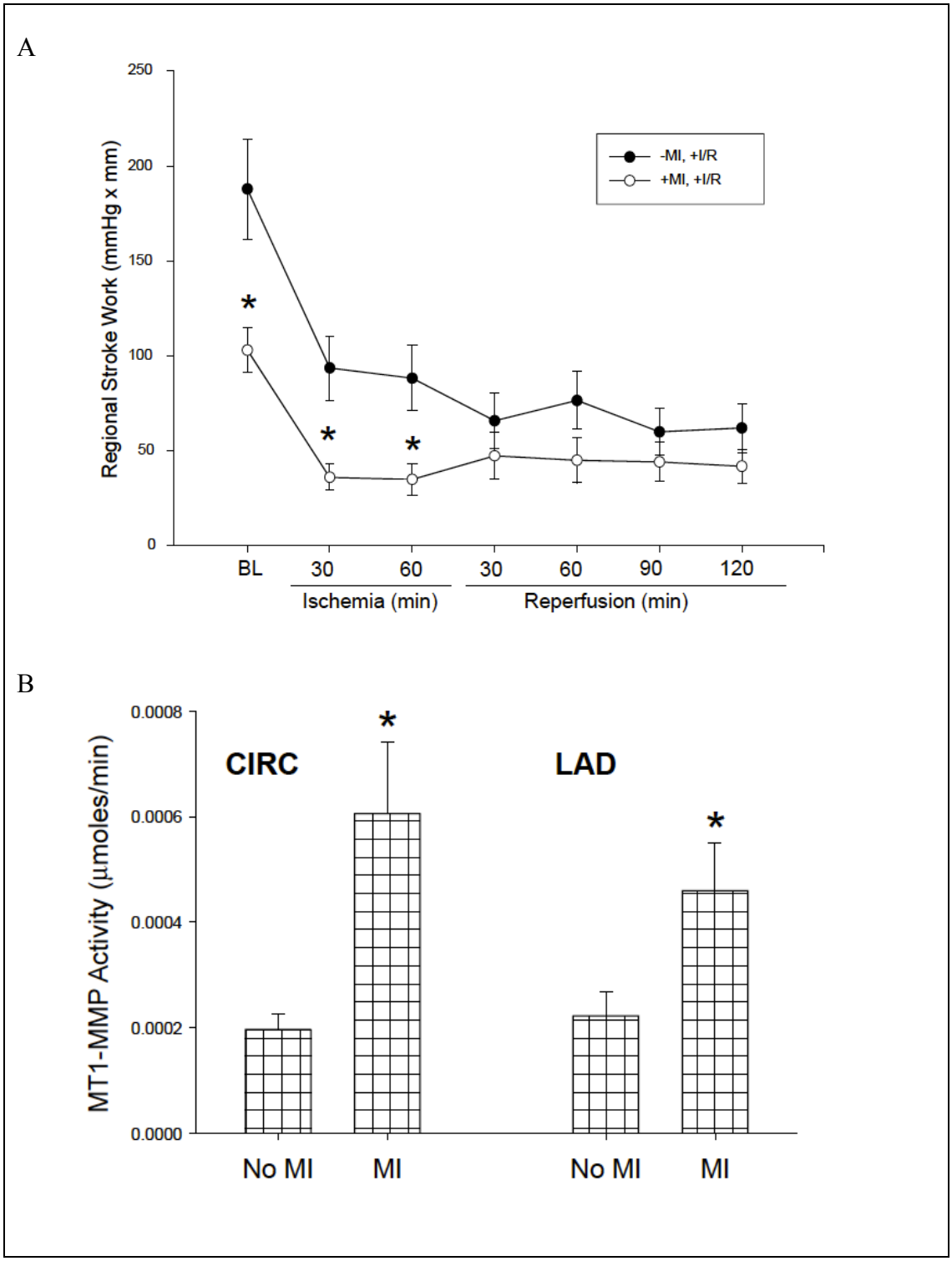


Figure 1.9 A. Regional stroke work in pigs without and with previous MI undergoing IR. B. MT1-MMP fluorogenic activity at baseline (BL).

A.Stroke work was decreased at baseline (BL) in animals with previous MI and remained decreased through peak ischaemia compared with animals without previous MI. B. In both circ (initial MI) and LAD (targeted for acute IR) regions, baseline MT1-MMP activity was elevated in animals with previous MI compared to animals without previous MI. [42]

1.3.4 Transgenic and pharmacological inhibition studies of MMP2 in heart disease

The detrimental effects of MMP2 on myocytes are reiterated in some ways in transgenic (knockout) and pharmacological inhibition studies. There are very few transgenic studies involving MMP2. Matsumura and co-workers studied wild-type (WT) and MMP2 knockout (KO) mice in a surgical MI model [43]. After induction of surgical MI, the WT mice were randomised to receive either a synthetic selective MMP2 inhibitor or vehicle alone. During the study period of 28 days, the survival of WT mice treated with MMP2 inhibitor and MMP2 KO mice were significantly higher compared to WT control group. The main cause of death in all the mice was cardiac rupture, which accounted for 38.5% of the mortality. The rupture threshold of the hearts was tested at 3 days after MI in the 3 groups and it was found that in WT control mice, the threshold was significantly lower by about 100 mmHg. MMP2 activity was confirmed to be inhibited in WT mice treated with the inhibitor and absent in the MMP2 KO group. ECM degradation in the groups with inhibited or absent MMP2 activity was suppressed compared to WT control group. In a different study, using the pressure-overload (induced by transaortic constriction) model, anterior and posterior wall thickness was significantly higher in WT mice compared to MMP2 KO mice [44]. Left ventricle end-diastolic pressure (LVEDP) was also significantly higher in the WT mice. Histology confirmed that myocyte hypertrophy and interstitial fibrosis induced by LV pressure overload were ameliorated in MMP2 KO.

There are more data available from the literature with pharmacological MMP2 inhibition studies to reinforce its negative role in cardiovascular diseases. Exogenous MMP inhibitors can be general or specific, and reversible or irreversible. A number of pharmacological agents have been identified that inhibit MMPs; these include 1,10-phenanthroline, marimastat, ilomastat and doxycycline [13, 45]. One potentially clinically useful inhibitor is doxycycline; it is the most potent inhibitor from the tetracycline class of broad-spectrum bacteriostatic antibiotics and its inhibitory effects are thought to occur independently, and at lower concentrations, to its antibacterial actions. It is the only drug acting as an MMP-inhibitor approved by the Food and Drug Agency (FDA) in clinical use as a treatment for periodontitis. Under experimental circumstances, doxycycline was found to inhibit MMP2 at a dose as low as 10 μM [35, 38]. It is possible

that doxycycline decreases MMP activity by reduction of enzyme activity and RNA stability and inhibition of transcription in long-term inhibition. In acute inhibition, it inhibits MMP by its zinc-chelating action.

In infarcted mouse hearts, at 4 days following the infarction, there is less increase in both the end-systolic and diastolic LV diameter at both mid-papillary and apical level with a broad synthetic MMP inhibitor, CP-471,474 (Fig 1.10) [46].

TABLE 2. Change in Echocardiographic Measurements From Day 1 to Day 4 in Infarcted Mice

	MMP Inhibitor (n=20)	Placebo (n=20)	*P*
Midpapillary measurements			
ΔED diameter, mm	−0.2±0.5	0.2±0.3	<0.01
ΔES diameter, mm	−0.3±0.4	0.1±0.4	<0.01
ΔM-mode FS, %	−3±13	−12±12	0.04
ΔED area, mm^2	−0.7±2.9	1.3±2.4	0.03
ΔES area, mm^2	−1.4±2	0.2±1.7	0.01
Δ2D FAC, %	−4±10	−9±13	0.19
Apical measurements			
ΔED diameter, mm	−0.1±0.3	0.2±0.3	<0.01
ΔES diameter, mm	−0.3±0.4	0.1±0.4	<0.01
ΔM-mode FS, %	0.1±14	−6±10	0.13
ΔED area, mm^2	−0.9±2.3	1.1±2.4	0.01
ΔES area, mm^2	−1.2±2.5	0.2±1.5	0.03
Δ2D FAC, %	−4±10	−7±17	0.48

ED indicates end diastolic; ES, end systolic; FS, fractional shortening; FAC, fractional area change; and 2D, 2-dimensional.

Figure 1.10 Change in parameters measured with echocardiography in infarcted mice Day 1 to Day 4. [46]

In a longer term study, mice were examined 4 weeks after surgical induction of MI [47]. The internal LV diameter and anterior wall thickness are preserved with MMP2 inhibition using doxycycline (Fig 1.11).

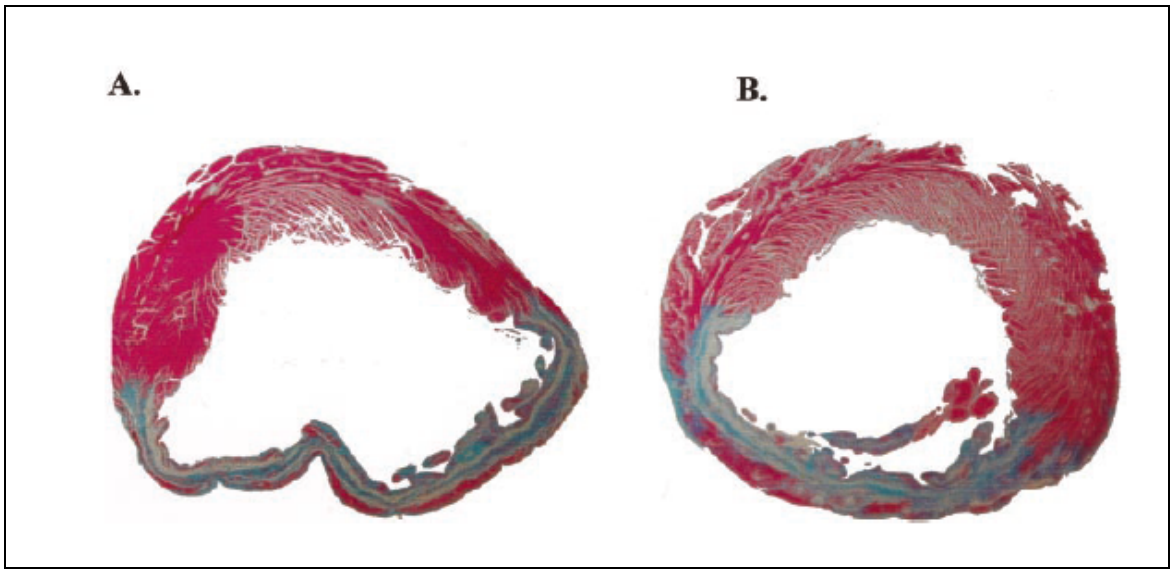


Figure 1.11 Representative ring segments from infarcted rat hearts in the absence (A) of or treated with doxycycline (B) for 4 weeks following infarction. [47]

Muscle tissues are seen as darker halftone, with scar tissue in blue.

MMP2 inhibition not only preserves the LV dimensions, but it seemed to attenuate the progressive LV dilatation associated with post MI remodeling and reduce the expansion of infarct size [48]. The consensus from other MMP2 inhibition studies seemed to concur that there is decreased acute LV remodeling, a lesser degree of change in LV dimensions, decreased myocardial wall stress and a lower risk of LV rupture [49-51].

However, there are also studies of prolonged MMP inhibition that suggest it may be associated with adverse outcome, probably due to its interference with wound healing and angiogenesis [52, 53]; a variety of MMP2 inhibitors, including doxycycline, were used in these studies. There are a few unresolved issues regarding MMP2 inhibition studies. The inhibitors were introduced at different time points during the protocol and the duration of treatment of MMP inhibitors varies between different studies. Tessone and co-workers started MMP inhibition day 1 post MI with subcutaneous doxycycline, with the treatment continuing for 9 days [53]. On the other hand, Spinale and co-workers used a synthetic MMP inhibitor, PD200126, which was only started orally 3 days after MI and the treatment continued for a total of 6 months [52].

In the setting of acute ischaemia-reperfusion studies as discussed in Section 1.3.3, various MMP inhibitors used (doxycycline, 1,10-phenanthroline, PD-16673) resulted in amelioration of mechanical dysfunction thought to be secondary to MMP2 [35, 37, 38]. MMP2 inhibition resulted in less myocardial injury in the rat hearts subjected to isolated heart perfusion and ischaemia-reperfusion as demonstrated by less leakage of creatine kinase (CK) into the coronary effluent, as well as protecting the integrity of the endothelial barrier [41]. The loss of intracellular target proteins of MMP2 such as α-actinin (part of the cytoskeletal structure) was also prevented with MMP inhibition, with a concomitant improvement in the mechanical function of the isolated hearts [54]. In isolated myocyte studies looking at the effects of peroxynitrite, $ONOO^-$, on contractile dysfunction, it was thought that $ONOO^-$ caused contractile dysfunction via activation of MMP2 [39]. Inhibition of MMP2 delayed the onset of contractile dysfunction in the myocytes when exposed to $ONOO^-$. This study using isolated and skinned myocytes further reinforced the concept that MMP2 exerts its detrimental effects on the hearts following acute ischaemia-reperfusion through a mechanism of action that is independent of its effects on the ECM.

Clamping of aortic inflow line during isolated heart perfusion in the studies mentioned [35, 37, 38], which caused global ischaemia, induced acute ischaemia-reperfusion injury discussed above. Such global ischaemia could occur clinically during systemic hypotension or may also be electively induced, as would occur during cardiac surgery. The duration of ischaemia determines the extent of molecular and cellular changes. These changes are initially reversible with full recovery of the function of the heart [55]. However, there will be a point when reversible changes become irreversible; even with reperfusion, there will be little or no recovery. In coronary artery bypass graft surgery (CABG), post-ischaemic myocardial dysfunction may cause complications such as MI, severe ventricular dysfunction, heart failure and death in about 10% of patients [56].

1.4 Myocardial protection and cardiac surgery

During cardiac surgery, global ischaemia is electively induced to facilitate a bloodless operating field. The aims of cardioprotection during elective global ischaemia are to re-

institute perfusion (or blood flow) to the heart as early as possible, thereby limiting the duration of ischaemia and to extend the duration of reversible injuries by slowing down the rate of development of ischaemic injury [55].

Over the years, various ways have been used in an attempt to protect the heart during this period of ischaemia. The first open heart surgery in human was performed in 1953; this was prior to the use of the cardiopulmonary bypass machine, and used systemic hypothermia to protect the heart [56]. In the 20 years following this landmark surgery, the concept of reversible chemical arrest (with the first known use of the term cardioplegia in 1957) was introduced in human and refined over the years. The ideal cardioplegia should induce rapid diastolic arrest, enhance myocardial protection, is easily reversible and has low toxicity [55]. One of the commonest cardioplegia solutions in use clinically today in the UK is the St Thomas' Hospital solution, which induces rapid diastolic arrest.

There are five elements of effective cardioprotection which formed the basis for much of the ongoing research work in improving cardioplegia [55]. Induction of rapid and complete diastolic arrest to reduce myocardial oxygen demand is fundamental. There are various mechanisms to achieve this. St Thomas' Hospital solution induced a depolarised cardiac arrest by elevating extracellular potassium concentration. Extracellular hyperkalaemia causes depolarisation of the myocyte transmembrane potential [55]. When membrane potential reaches about -65 mV, the voltage-dependent sodium channel is inactivated and thus abolished the generation of action potential, resulting in diastolic arrest of the heart. However, further depolarisation of membrane potential to around -45 mV will in turn activate the slow calcium channel leading to influx of calcium ions and eventually intracellular calcium overload. Hence, the beneficial effect of hyperkalaemia is confined to a narrow window of membrane potential. Yet, even within this narrow window of depolarisation, there is ongoing ionic imbalance due to window currents. Therefore, ongoing research is focused on other mechanisms to induce arrest such as polarised arrest or inhibition of calcium influx [57]. The second way to decrease myocardial oxygen consumption is by inducing hypothermia. With 10°C drop in temperature, enzyme activity is decreased by about 50% [58]. However, although effective at minimising oxygen consumption, there are disadvantages such as ionic disturbance, alteration to microvasculature and development

of contracture when the temperature drops below 5°C. The third element of cardioprotection is to minimise the ischaemic effects with anti-ischaemic agents [55]. Various strategies such as using blood as a vehicle of cardioplegia delivery, oxygenation of cardioplegia, buffering capacity of cardioplegia and additives such as anti-oxidants are also used. The fourth strategy is to optimise reperfusion conditions to maximise recovery, by reducing oxidative stress and production of free radicals. Supplementation with anti-oxidant enzymes and pharmacological inhibition of free radicals are such examples. Lastly, the vasculature, especially endothelium, and conducting tissues should also be protected during ischaemia to enhance post-ischaemic recovery of the heart.

Despite major advances made in myocardial protection strategy, the ideal cardioplegia has yet to be found. There is still a high prevalence of inadequate protection during cardiac surgery, with myocardial stunning occurring in 20-80% of patients post-operatively [56]. If anything, in the years to come, the incidence will only increase due to the changing profile of patients undergoing cardiac surgery. Patients are increasingly in the higher risk group, with urgent or emergency surgery becoming more common, especially in patients with recent ACS. The search for better cardioprotection during surgery continues, especially as better cardioprotection not only translates to better immediate outcome in terms of in-hospital mortality, but it will also impact the long term outcome and survival after cardiac surgery [59].

1.5 Acute coronary syndrome (ACS)

Acute coronary syndrome (ACS) constitutes the largest indication for acute hospital admissions. Advances in diagnostic technology have enabled accurate diagnosis and stratification of myocardial infarction, leading to timely and appropriate reperfusion. However, with increased diagnostic capabilities, it also emerged that rather than a single entity, ACS comprises a very diverse group of patients with different risk profiles and outcomes (Fig 1.12) [6, 60]. ACS is a spectrum of clinical conditions, ranging from frank ST elevation myocardial infarction (STEMI) to non-ST elevation infarction (NSTEMI) to unstable angina. The underlying pathophysiology in ACS was discussed in Section

1.2. The nature and composition of the plaque and the subsequent inflammatory processes that follow are now thought to exert a significant contribution to the disease process. STEMI is usually associated with a major thrombotic event involving the coronary artery, where the artery is totally occluded by fibrin-rich thrombus. On the other hand, in NSTEMI, the coronary artery tends to be partially occluded by platelet-rich thrombus [61]. The diagnosis of ACS is established on the basis of typical clinical presentation, electrocardiographic evidence of ischaemia and the presence of biomarkers of myocardial injury [6, 62] (Fig 1.12). The early and mid-term clinical consequences of ACS are serious with high mortality, further ischaemic episodes and hospital readmission as well as other thromboembolic event such as cerebrovascular insults. Hence, the aim of treatment is timely reperfusion with adequate analgesia, adequate arterial oxygenation and relief of ischaemia.

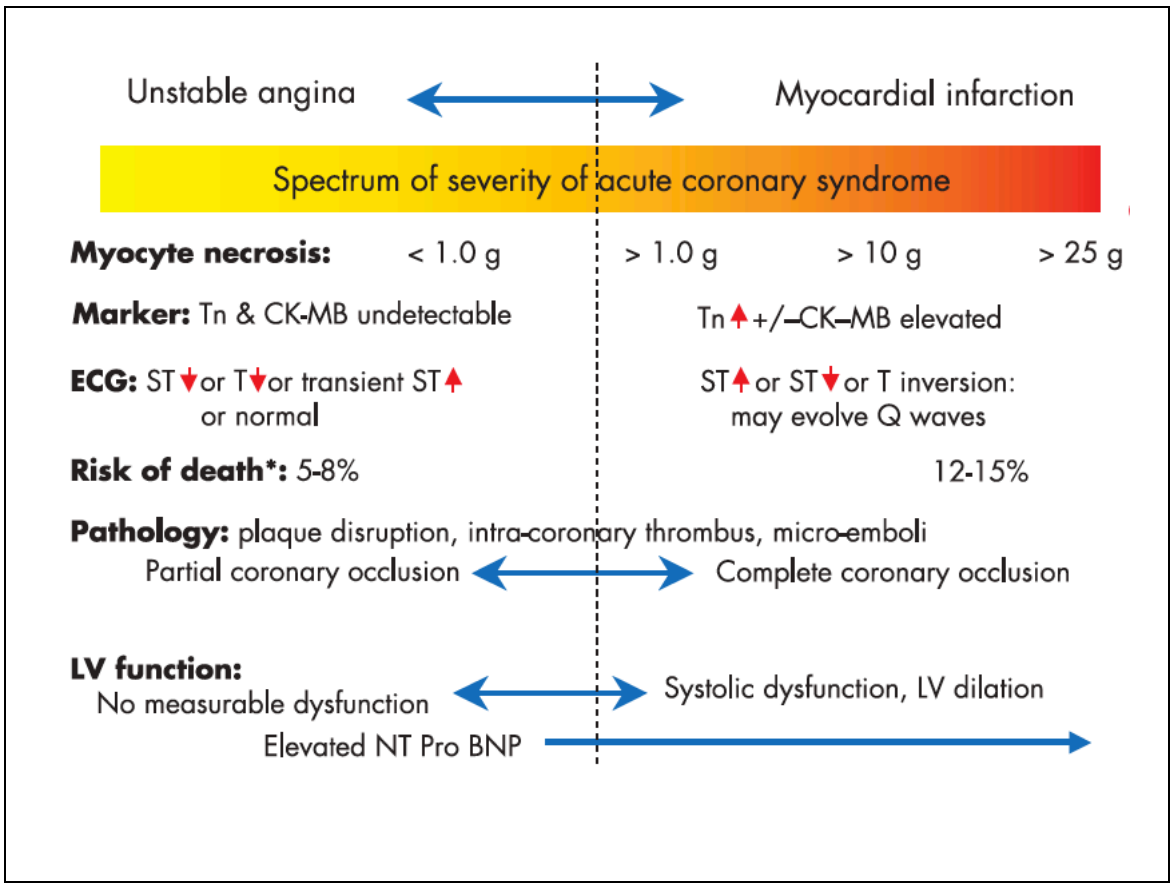


Figure 1.12 Diagram illustrating the spectrum of ACS.

ACS ranges from unstable angina to MI, with its associated biomarkers, electrocardiographic and haemodynamic characteristics. [6]

In patients with STEMI, due to the complete occlusion of the coronary artery, there is a high risk of morbidity and mortality. Prompt and effective reperfusion in the occluded vessel is the main aim of treatment [63, 64]. Reperfusion in STEMI is preferably by primary percutaneous coronary intervention (PCI) with stents or angioplasty [65]. However, if a hospital capable of primary PCI is not available within 60 min, then medical thrombolysis with clot-busting drugs such as streptokinase is the alternative option. Surgery is usually considered a rescue therapy if thrombolysis or PCI has failed, or if there are associated complications such as ruptured septum or chordae tendinae of the mitral valve resulting in haemodynamic compromise. Hence, surgery is best delayed until at least a few days after the patients are stabilised, as the associated surgical mortality is high [66].

In patients with NSTEMI or even unstable angina, optimal treatment strategy remains an area of great debate. The cardiovascular risk in this group of patients continues with time as a result of further thrombotic event. Hence, the principle of treatment is to relieve the ischaemia and prevent further myocardial injury. There are 2 major issues to be resolved: the type of intervention and the timing of the intervention. Due to the heterogeneity of the patients, risk stratification with scoring systems remains an important consideration. In the newly published collaborative European Society of Cardiology/European Association for Cardiothoracic Surgery (ESC/EACTS) guidelines, the GRACE score is the preferred system [64, 67, 68]. The scoring system was developed in 1999 from the collaborative Global Registry of Acute Coronary Events (GRACE) and takes into account factors such as the age of the patient, the heart rate, systolic blood pressure, creatinine level, degree of pulmonary oedema, presence of cardiac arrest, ECG changes and cardiac biomarkers on hospital admission. The early invasive strategy is strongly recommended in high and medium risk groups, supported by Level A evidence [64]. The mode of intervention should then be dictated by the coronary anatomy, preferably in a multi-disciplinary setting. Hence, in some patients, urgent or emergency coronary artery bypass surgery may be the optimal treatment as part of the management of their acute coronary syndromes. Besides, despite aggressive non-surgical revascularisation, there may be ongoing ischaemia, or there may be other complications such as valvular insufficiency, ventricular septal defects or even complications

following PCI necessitating emergency surgery. Surgical mortality in urgent or emergency operation is very high, at up to 17% [69].

In the 6th National Adult Cardiac Surgical Database Report published by the Society for Cardiothoracic Surgery of Great Britain and Ireland, the crude mortality in urgent or emergency coronary artery bypass graft surgery is 8% compared to 1% when the procedure is performed electively (Fig 1.13). Other surgical series have also demonstrated higher surgical mortality and morbidity associated with urgent coronary artery bypass surgery [66, 69].

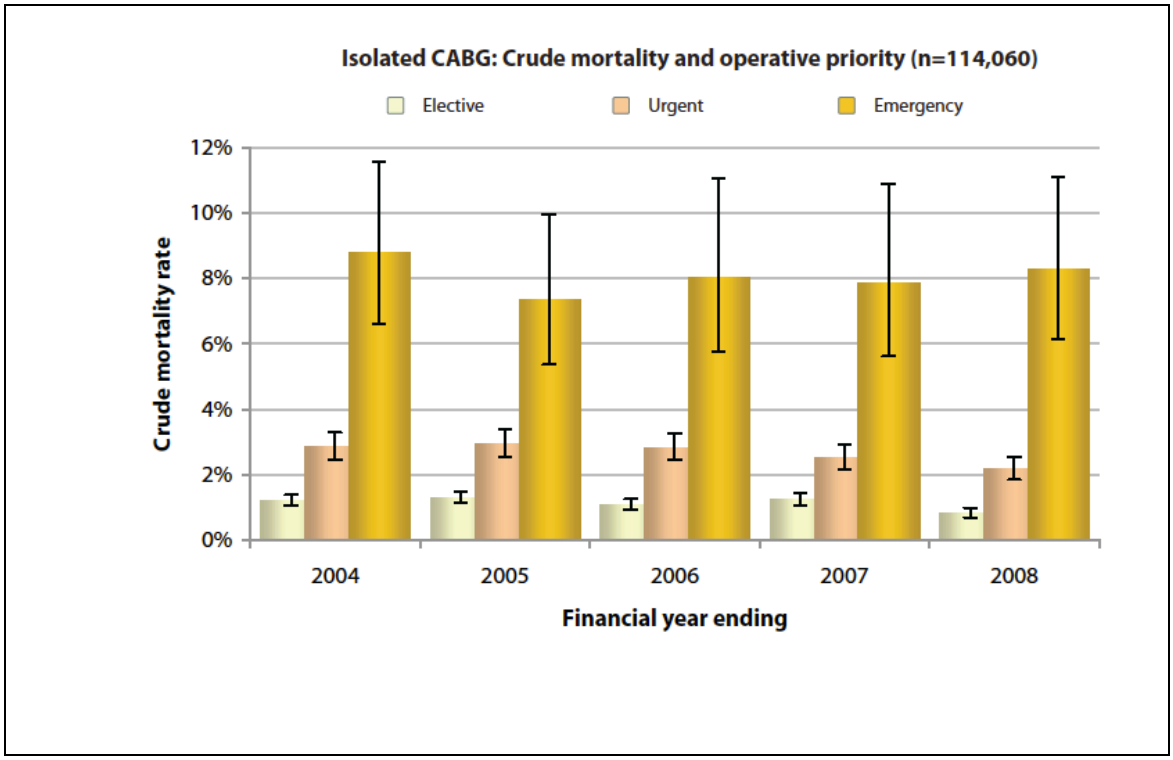


Figure 1.13 Data from the 6th National Adult Cardiac Surgical Database Report 2008. The crude mortality rates for isolated CABG based on urgency of the operation. [70].

If the timing from MI to surgery is scrutinised further, it is obvious that surgery within 24 hours of admission with MI is at least 3-fold higher compared to when surgery was carried out more than 24 hours after MI (Fig 1.14).

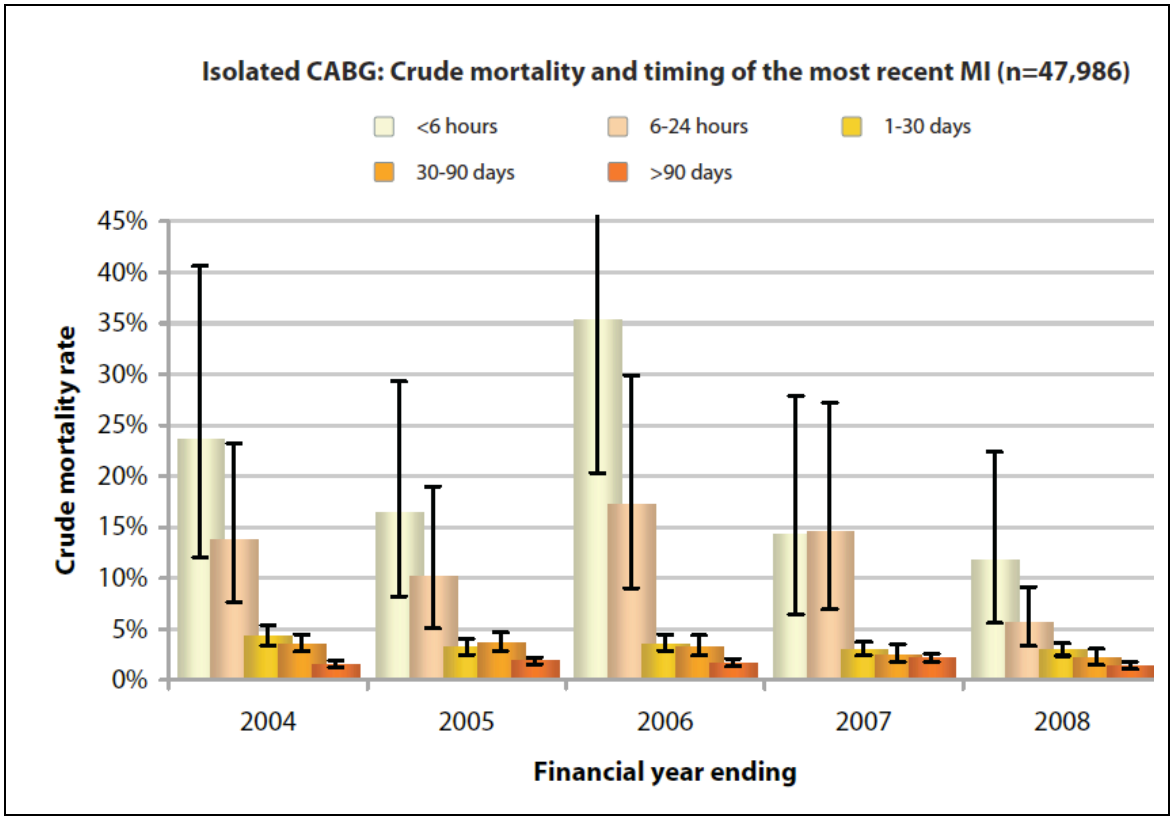


Figure 1.14 Data from the 6th National Adult Cardiac Surgical Database Report 2008.

Crude mortality rate in isolated CABG based on the timing of the most recent MI, with highest mortality in surgery carried out within 24 hours of MI. [70]

In a registry study from California, similar results were observed. The mortality was 8.2% when surgery was performed within 24 hours of MI [71]. The mortality decreased with increasing interval from MI to surgery to reach a low of 3% at 3 days after MI (Fig 1.15). From the 3rd day to 7th day, there was a suggestion of slight gradual increase in mortality before a sharp increase in patients operated on after 14 days of MI. This probably represents a group of patients who were very ill and attempts made to stabilise the patients medically, with surgery carried out as a last resort or salvage operation.

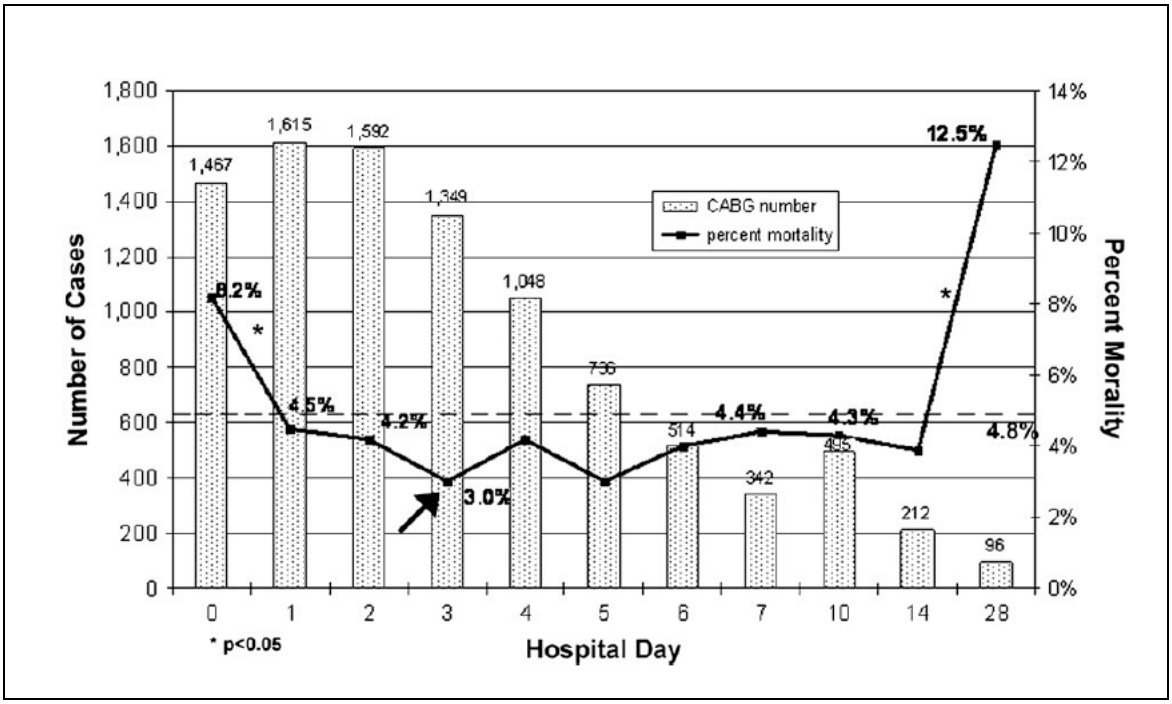


Figure 1.15 CABG volume and mortality over time

Distribution of CABG volume (left y-axis, no of cases/day) and % mortality (right y-axis) per day of hospitalisation. Dashed horizontal line represents total mortality over the study period (4.8%). [71]

Although these reports use crude mortality rates and are not from randomised controlled studies, it provides a snapshot of the experience in cardiac units in a realistic setting.

The pathophysiology of acute ischaemia-reperfusion injury, either regional acute myocardial infarction or global ischaemia as electively induced during cardiac surgery, has been studied extensively both at the cellular and molecular level [55, 72]. As a result, various therapeutic interventions, such as cardioprotection during cardiac surgery as discussed in Section 1.4, have greatly enhanced and improved the clinical management and outcome of these patients. However, regional ischaemia followed rapidly by global ischaemia is rarely studied together in the experimental setting, although it is likely that acute regional ischaemia and subsequent global ischaemia will result in greater myocardial dysfunction than either situation on its own, despite the cardioprotective techniques that can be incorporated during cardiac surgery. Clinically, this scenario is clearly depicted by patients with acute myocardial infarction, which results in acute ischaemia of the territory supplied by the coronary artery involved, who subsequently

undergo urgent coronary artery bypass surgery, when their myocardium is subjected to further global ischaemia. An initial ischaemia may result in upregulation of bioactive molecules, with a consequent activation of various proteolytic pathways and proteases [73-75]. The upregulation of these bioactive molecules may occur even in the region remote to the infarcted or ischaemic zones. The pathways are then primed to respond when a second ischaemia-reperfusion injury occurs such that the resultant left ventricular dysfunction is exacerbated [42]. There are many factors that are involved in the pathophysiology of myocardial ischaemic injury; these include mitochondrial dysfunction leading to increased oxidative stress, reduced contractility associated with dysfunction of the sarcoplasmic reticulum Ca^{2+}-ATPase and cellular and mitochondrial ionic changes [76]. There is also increasing evidence that extracellular matrix degradation is involved in cardiac dysfunction associated with myocardial ischaemia [77, 78].

Since the seminal discovery of MMPs by Gross and Lapiere [9, 12], research into the various biological and pathological roles of MMPs has rapidly expanded over the decades. In cardiovascular diseases, it has become clear that MMPs, especially MMP2, play a significant role not only in long term remodeling processes, but they are also very much involved as key players in acute processes such as ischaemia-reperfusion.

There is an emerging and significant clinical scenario of patients with acute coronary syndrome, who may require urgent cardiac surgery within one week of the onset of ACS. There is currently ongoing debate about the optimal revascularisation strategy in this group of patients. As mentioned earlier, the morbidity and mortality in this group of patients are invariably higher. Ongoing research into better cardioprotection for this group of patients is required to improve the outcome. Even in elective cardiac surgery, with a much lower mortality, current cardioprotection with cardioplegia during surgery is not optimal. The heart is still subjected to oxidative stress with inadequate protection of tissues such as the microvasculature. The role of MMP2 in this circumstance has not been established, although there is supporting evidence that link MMP2 with oxidative stress [79] and that MMP2 regulates vascular tone, and has both vasodilatory and vascoconstrictive effects [13]. MMP2 inhibition during cardiac surgery may provide an additional adjunct in protection of myocardium, optimisation of reperfusion condition by reducing the effects of oxidative stress and protection of the endothelium of coronary vasculature.

1.6 Hypothesis, aims and objectives

We hypothesise that increased matrix metalloproteinase (MMP) activity, induced by an acute ischaemia-reperfusion event in the heart, is associated with the resultant cardiac dysfunction. Since cardiac surgery imposes an additional ischaemia-reperfusion burden, effects of MMP activity will be exacerbated. Cardioplegia protection, with or without MMP inhibition will improve post-ischaemic cardiac function.

The aims of this study are to elucidate the role of MMP2, in a laboratory setting, where regional infarction is initially induced in laboratory rat hearts, and subsequently after a week, the infarcted hearts are then subjected to global ischaemia, as would occur during cardiac surgery. The isolated Langendorff perfusion model would be used as a model to study the effects of global ischaemia. This, we hope, will contribute towards improving cardioprotection in patients undergoing cardiac surgery after acute coronary events.

Our specific objectives are therefore to determine:

1. What is the additional effect of global ischaemia-reperfusion on the infarcted hearts, as would occur during cardiac surgery?
2. What is the profile of MMP2 activity in the infarcted hearts during the additional global ischaemia-reperfusion during isolated heart perfusion?
3. What is the influence of cardioplegia protection (with or without inhibitors of MMP) during the global elective ischaemia-reperfusion on the mechanical function of the heart?
4. What is the optimal timing for the administration of MMP2 inhibitors post-myocardial infarction to achieve the maximal benefit?

2 GENERAL METHODS

The general experimental techniques used in the studies are described in this chapter. Where there were specific alterations to the standard technique or if the method was only used once, it is described in detail in the relevant chapters. Specific protocols pertaining to certain studies are also described in the relevant chapters. The animals used in these studies were adult (10-week old), male Wistar rats, weighing between 250 to 350 g in body weight. All animal experiments were performed in accordance with the United Kingdom Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office.

2.1 Surgery and anaesthesia

Animal experiments were performed in keeping with the standards and conditions set out by King's College London and the Home Office Animals Scientific Procedure Act (1986). All proposed procedures and protocols were also discussed with the veterinarian prior to commencement. All procedures carried out were included within the schedule of my personal license (PIL 70/22124) and the departmental project licences (PPL 70/6446 and PPL 70/6635).

Rats were housed in the Biological Services Unit (BSU) on site and within the Waterloo campus of King's College London. The ambient temperature was maintained at 24°C, with 55-65% relative humidity, 12-hour alternating light-dark cycle and with free access to food and water. Recovery surgery and anaesthesia were performed within a dedicated procedure room in the BSU at the Waterloo campus, with strict asepsis. The animals were recovered for 7 days prior to isolated Langendorff heart perfusion studies.

2.1.1 General anaesthesia

Recovery surgery and procedures were performed under inhalational anaesthesia with isoflurane. Isoflurane is a volatile halogenated agent, which has a more favourable cardiovascular profile compared to other anaesthetic agents, with less myocardial sensitisation to endogenous catecholamines and other related compounds [80].

Furthermore, recovery of the animal is faster with isoflurane. Isoflurane was delivered in 100% oxygen at a flow rate of 3 L/min via a vaporiser. Induction of anaesthesia was carried out by placing the rat in an induction chamber and then transferring to an operating table with a homeothermic plate, using an open circuit system with a nose-cone (fashioned from a 10 ml syringe). Where thoracotomy was required, invasive ventilation was then used with endotracheal intubation as described below.

The depth of anaesthesia was determined by assessing respiratory rate and nociception with toe pinching. Intervention was only carried out once these reflexes were abolished.

2.1.2 Invasive mechanical ventilation with endotracheal intubation

There are various ways described by different principal investigators within our department regarding endotracheal intubation of rodents. Essentially, it could be either direct vision of the vocal cords using a paediatric laryngoscope or surgical dissection of the trachea to visualise the endotracheal tube in the trachea directly. After long consideration, given the instruments that were readily available, with the position of the animal to optimise surgical procedure, I decided that surgical dissection of the neck to visualise the endotracheal tube was best suited for this project. Intubating with a laryngoscope would require the animal to be positioned initially with the head towards the operator, and subsequently changing the position of the animal for the surgical procedure. This was not logistically feasible given the way the anaesthetic circuit is set up on the operating table.

With the animal placed supine on the operating table, ventilated with the nose-cone system, dissection was carried out to expose the trachea. The larynx is bordered by the epiglottis ventrally, aryepiglottis laterally and arytenoid cartilage dorsally. The wall of the laryngeal vestibule is flexible. The glottis is bounded by elastic vocal ligament and rigid cricoarytenoid ligament [81]. The elastic epiglottis is shaped like a grooved triangular leaf in a straight caudal base and long apex. Pointed and curved cuneiform processes arise near the base laterally.

A small vertical incision was made in the midline in the neck. The subcutaneous tissue was dissected to expose sternohyoideus muscle, which was separated and retracted to expose the trachea (Fig 2.1). With the rat supine, the tongue was gently retracted with a pair of forceps to open the epiglottis.

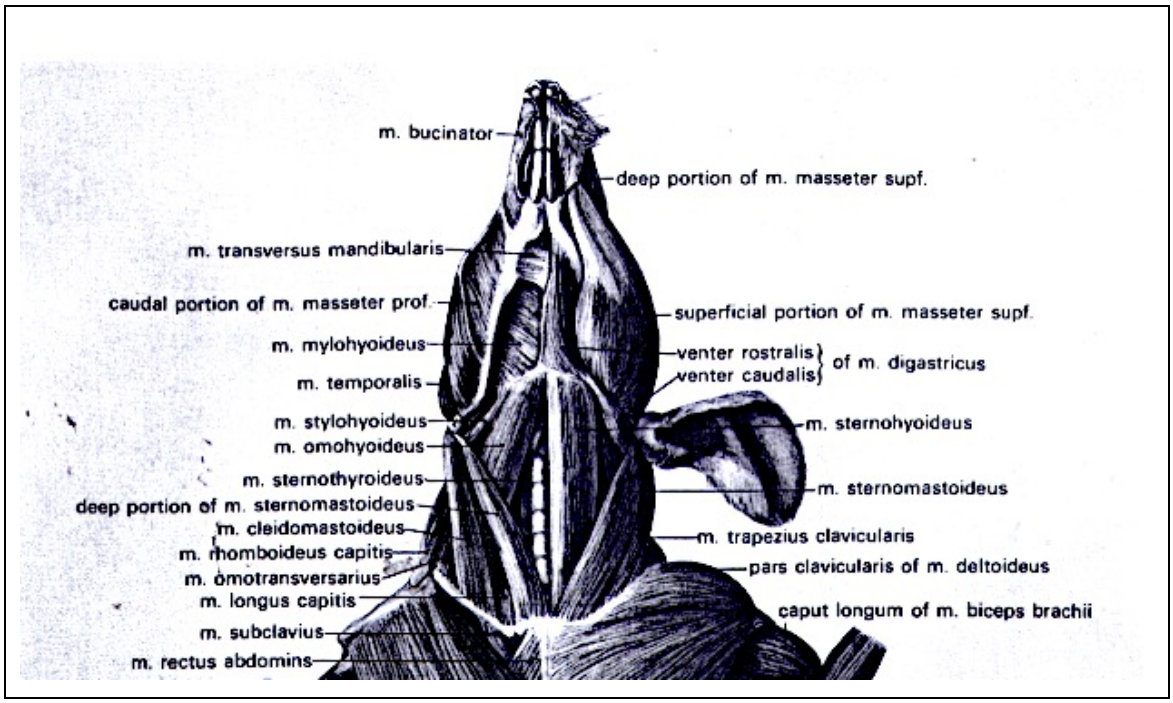


Figure 2.1 Superficial and deep anatomy of the neck of a rat [81]

An endotracheal tube (ETT) (which was fashioned using a 14G cannula) was then gently advanced endotracheally and under direct vision into the trachea (Fig 2.2). The position of the ETT was maintained at just above the carina. This is to prevent injury to the carina, which may result in tension pneumothorax, causing the death of the animal shortly after surgery. The 14G cannula formed a reasonably tight seal with the trachea to allow satisfactory ventilation. The animal was ventilated using the 7025 rodent ventilator (Ugo Basile) with a respiratory rate of 50, and tidal volume of 2 ml.

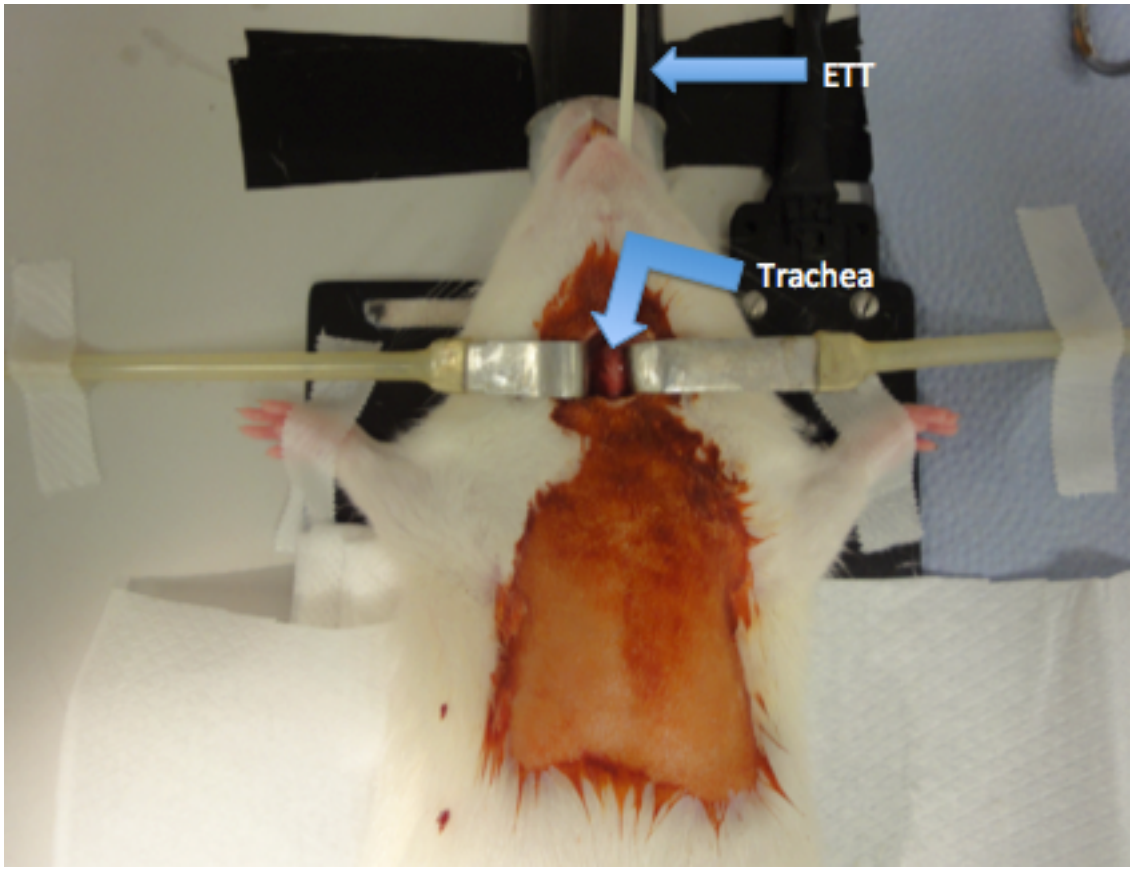


Figure 2.2 Insertion of ETT under direct vision.

The neck incision was retracted to expose the trachea. Under direct vision, the ETT was inserted into the trachea.

At the end of the surgical procedure, it is important to ensure adequate expansion of the lungs to optimise recovery of the rat. This was achieved by increasing the tidal volume to 3-4 ml with positive end expiratory pressure (by submerging the efferent tubing of the exhaust port in water). Isoflurane was discontinued and ventilation maintained with 100% oxygen. The respiratory rate was gradually decreased to 35-40 breaths/min until spontaneous ventilation resumed and nociceptive reflexes restored, at which point the animal was extubated. The animal was then recovered in a hot-box at 24°C overnight.

2.1.3 Anterior thoracotomy and ligation of left anterior descending (LAD) artery

Prior to performing the surgical procedure, it was essential to understand the anatomy of the coronary arteries, particularly the left coronary artery, in the rodents. The left coronary artery originates from the aorta, in contact with the left margin of the pulmonary cone, at about 1 mm from the insertion of the left atrial appendage (Fig 2.3) [82]. The left coronary artery is predominant and supplies the left ventricle. The coronary artery lies beneath the epicardium, buried in the myocytes. Hence, it is important to ensure the sutures are placed at an adequate depth to ligate the LAD effectively [83].

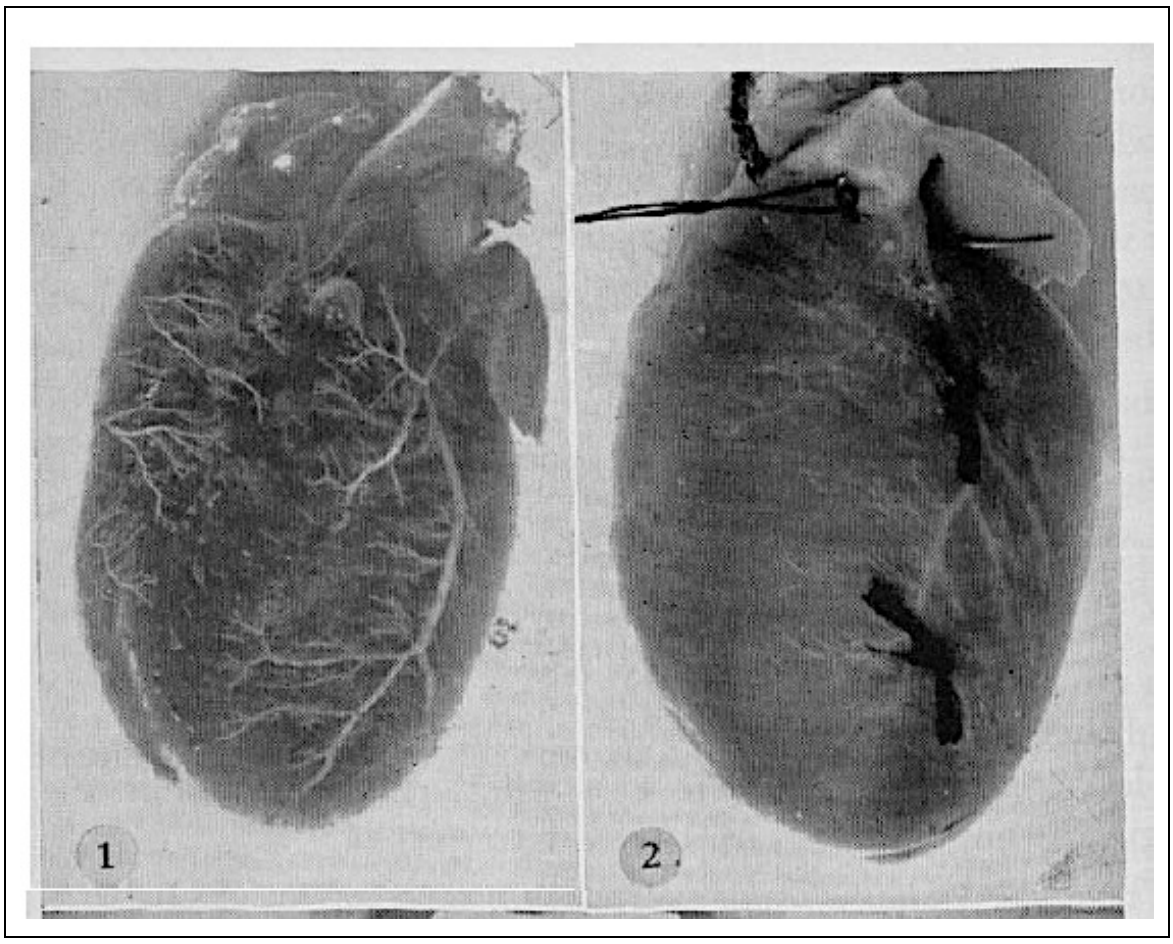


Figure 2.3 Anterior aspect of a rat heart. The coronary arteries of the heart were injected with lead oxide in latex (Picture 1). In Picture 2, a curved needle had been introduced between the pulmonary cone and the insertion of the left atrial appendage, to demonstrate the position for occlusion of LAD. Additional ligatures were also shown in the picture to show alternative positions to place the ligature more distally along the LAD, to produce smaller infarct if needed. [82]

In order to learn and master the technique of LAD ligation, time was spent discussing the technique with various experts and by observing the way the procedure was carried out in murines. The initial technique used was a result of the various discussions with modification from that observed in murines.

The final technique was modified after careful study of Selye's description of that used for surgical occlusion of coronary vessels in rat [82]. Once the animal was anaesthetised, the animal was secured on the homeothermic plate to maintain the core body temperature at 37°C (ADinstruments ML295/R). The fur from the thorax was then clipped with an electric shaver. Betadine was used to sterilise the thorax and surgery was performed using full aseptic technique. Clean and sterile drapes were used to cover the abdomen and on the side to provide a sterile operating field. A lateral skin incision along the ribs was made, starting just below the lower border of the forelimb, from the left mid-axillary line to just slightly to the right of the sternum. This was modified from the original technique described; Selye described a midline incision along the sternum, but lateral skin incision provided better access. The pectoralis muscle was then divided to the sternal attachment to reveal the sternum and intercostal muscle. At this stage, the respiratory rate is decreased down to 25-30 and tidal volume decreased to about 1 ml to visualise the beating heart so that the optimal intercostal space was used to enter the pleural cavity. Blunt scissors were used to carefully dissect the intercostal muscles, about 1 mm from the sternal edge to puncture the pleura (Fig 2.4). This avoids dissecting the internal mammary artery that runs along the sternum, which may result in massive haemorrhage. Respiratory rate and tidal volume were kept at a minimum to decrease the likelihood of injury to the lungs during puncture of the pleura. Once the pleural cavity was entered, the incision was widened gradually with the scissors. The pleura was carefully incised laterally to improve access to the heart. Carefully, the rib was cut using sharp scissors about 1.5 mm from the sternal edge. Haemostasis was achieved using electrocautery as the intercostal bundle would be invariably cut. Using electrocautery, it was possible to minimise the amount of bleeding. The incision was widened by dividing the intercostal muscles below this rib.

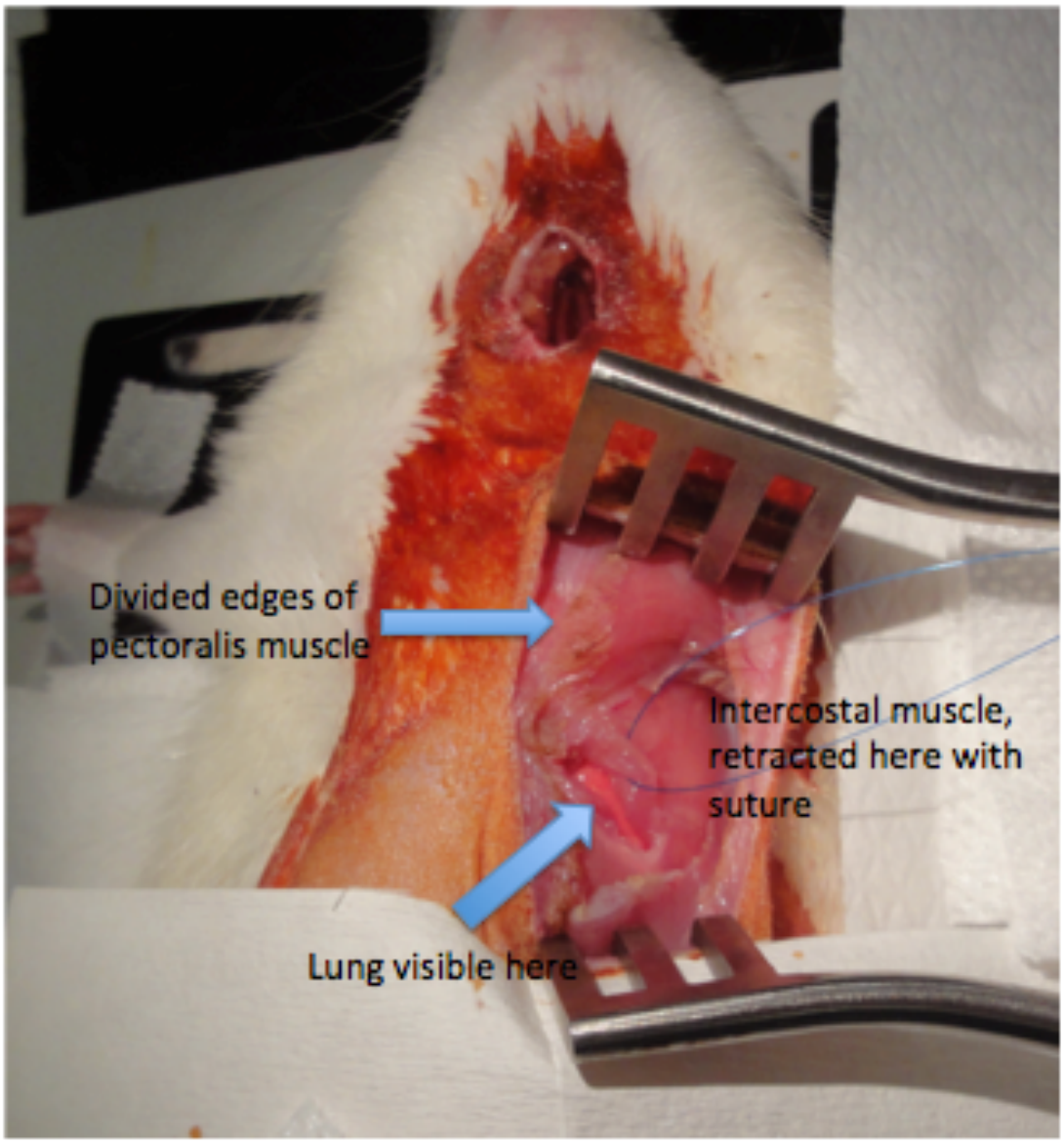


Figure 2.4 Anterior thoracotomy to expose the heart.

Lateral skin incison to expose underlying pectoralis muscle, which was separated and blunt dissection was carried out to enter the pleural cavity. The left lung was visible in this view.

Next, the pericardium was carefully perforated to visualise the heart. A pair of forceps was used to steady the cut sternal edge of the rib and, with the blunt end of another pair of forceps in the pericardial cavity, the heart was exteriorised with pressure on the right thorax (Fig 2.5). Once the heart was exteriorised, a suture was placed between the point of entry on the left margin of the pulmonary cone and the middle of an imaginary line

connecting this point with the closest point on the insertion line of the left atrial appendage. The suture used was 5/0 prolene with blunt needle.

Figure 2.5 Exteriorisation of the heart

The heart was exteriorised to enable placement of LAD suture safely.

Once the suture was placed, the heart was returned to the thorax before tying the suture. This was to ensure that the duration the haemodynamics of the heart were affected by the position of the heart outside the chest was as short as possible. When the suture was

tightened, there was visible evidence of ischaemia as the myocardium turned pale. Haemostasis was then achieved. 5/0 prolene was used to approximate the ribs together. The respiratory rate and tidal volume were restored back to 50 breaths/min and 3 - 4 ml mentioned previously. Positive end expiratory pressure (PEEP) of 1 cm water was also applied at this stage to ensure adequate lung expansion prior to approximating the ribs. Once the ribs were approximated, the PEEP was removed and tidal volume decreased back to 2 ml. The divided muscles were then approximated and during the process, intermittent pressure on the left thorax was applied to expel any residual air. The skin incision was closed with 5/0 prolene. Betadine was used to clean the wound prior to skin closure.

Normal saline (2 ml) and analgesia (vetergesic 0.2 mg/kg) were given to the animal intraperitoneally during recovery. Isoflurane was switched off and the animal extubated once spontaneous respiration resumed. The animal was then recovered in a hot-box at temperatures between 24°C to 28°C overnight with free access to food and water.

2.2 Isolated Langendorff heart perfusion system

The rat was anaesthetised with 0.8 -1.0 mls of 30% pentobarbitone (diluted in 0.9% saline) intraperitoneally. Once the animal was in deep anaesthesia, determined by the loss of pain reflex to toe-pinching, 0.1 ml of 1,000 U/ml of heparin was injected into the femoral vein.

Scissors were used to make a clamshell incision across the chest, with division of the diaphragm to expose the mediastinum. The heart was then rapidly excised and immersed in cold Kreb-Henseleit solution. The aorta was then cannulated on the Langendorff set-up and secured using 3/0 silk suture. The heart was cannulated within 30 s of excision. Once cannulated, the heart was retrogradely perfused with Kreb-Henseleit buffer (composition in mM: NaCl, 118.5; $NaHCO_3$, 25.0; KCl, 4.75; $MgSO_4$, 1.19; KH_2PO_4, 1.18; Glucose, 11.0 and $CaCl_2$ 1.4) at 100 cm H_2O at 37°C, continually gassed with a mixture of 95% oxygen:5% carbon dioxide. The suspended heart was also immersed in a warm fluid bath to maintain the temperature at 37°C (Fig 2.6).

An incision was then made to the left atrium and a water-filled balloon (which was fashioned from clingfilm to fit the left ventricle) was inserted through the mitral valve into the left ventricle [84]. The balloon was connected to a pressure transducer, which allows real-time measurement of the mechanical function of the heart. The balloon was inflated to achieve an end-diastolic pressure of 5-10 mmHg. The heart rate, left ventricular systolic pressure (LVSP) and end-diastolic pressure (LVEDP) was monitored and acquired continuously and stored using LabChart 7. The left ventricular developed pressure (LVDP) was determined by LVSP minus LVEDP. The heart was allowed to stabilise for 20 minutes before starting the experimental protocol. Exclusion criteria were applied during this equilibration period; any hearts that did not achieve a LVDP of ≥100 mmHg, or if the coronary flow (CF) was ≥20 ml/min (implying a leak within the perfusion system) or ≤6 ml/min (implying a perfusion problem) were excluded.

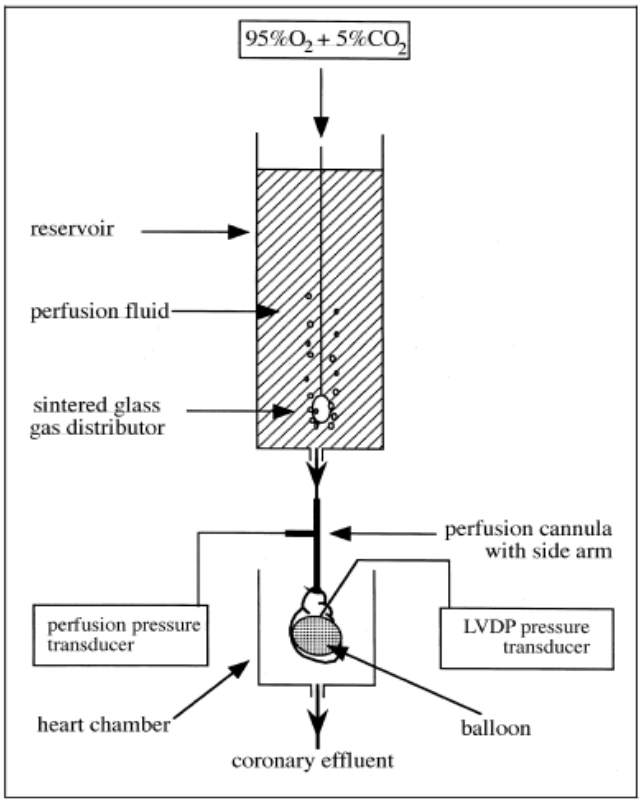


Figure 2.6 Schematic diagram of the isolated Langendorff heart perfusion, constant pressure system.

All parts of the perfusion hardware are water-jacketed with warm circulating water to maintain constant temperature [85].

A second reservoir attached to the side-arm of the perfusion rig allowed any experimental drug to be administered to the heart. Global ischaemia was induced by clamping the aortic in-flow line. Occasionally, during reperfusion, the heart may develop ventricular fibrillation. If this happened, the heart was defibrillated back to sinus rhythm.

2.3 Extraction of proteins from rat heart tissue

With any ex vivo assays of MMP2 activity, sample preparation is crucial. Care was taken to avoid adding reagents that could alter endogenous MMP activity such as common reducing agents (e.g ethylenediaminetetraacetic acid, EDTA, and dithiothreitol, DTT). Hence, some advocate avoidance of such compounds in extraction buffers [86]. On the other hand, without reagents such as DTT, the extraction process may not be adequate to yield sufficient proteins for analysis. This may explain why most studies were carried out with a small amount of such reagents [35, 37, 79]. Therefore, it was crucial to first of all determine the optimal composition of extraction buffer for use throughout these studies.

At the end of the isolated Langendorff perfusion protocol, hearts were freeze-clamped in liquid nitrogen, the heart samples were ground into a powder form in liquid nitrogen using a mortar and pestle, and subsequently stored at -80°C until use.

A sample of powderised heart (50 mg) was used with 150 μl of lysis buffer; 2 different lysis buffers were tested initially. A total of 30 heart tissue homogenates were used with buffer A and 42 with buffer B.

Composition of lysis buffer A (in mM):

β-glycerophosphate 20, sodium fluoride 50, benzamidine 10, sodium orthovanadate 0.2, Triton X-100 1%, Complete protease inhibitor tablet (Roche)

Composition of lysis buffer B (in mM):

β-glycerophosphate 20, sodium fluoride 50, benzamidine 10, sodium orthovanadate 0.2, DTT 1, Triton X-100 1%, Complete protease inhibitor tablet (Roche)

The samples were mixed with ice-cold buffer in 1.5 ml eppendorf tubes. A micropestle was used to mix and break down the tissues. The sample was then vortex mixed to allow thorough mixing. After about 5 min to allow for the extraction process, the samples were centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was collected for use in the Bradford assay to determine its protein content. The Bradford assay is described in Section 2.4. The mean protein concentration in the supernatant from the different buffers was compared using Student's t-test. A value of $p<0.05$ was considered statistically significant.

The mean protein concentration (μg/μl) when buffer A was used was 2.3±0.1 (95% CI 1.8-2.7), which was significantly ($p<0.0001$) lower than the equivalent extraction concentration of 22.0±0.8 (95% CI 18.7-24.4) when buffer B was used (Fig 2.7). This showed that a reducing agent, which linearises the protein structure making it easier for extraction, is required to improve protein extraction from tissues. Hence, in subsequent analysis, lysis buffer B was used for homogenisation of the hearts.

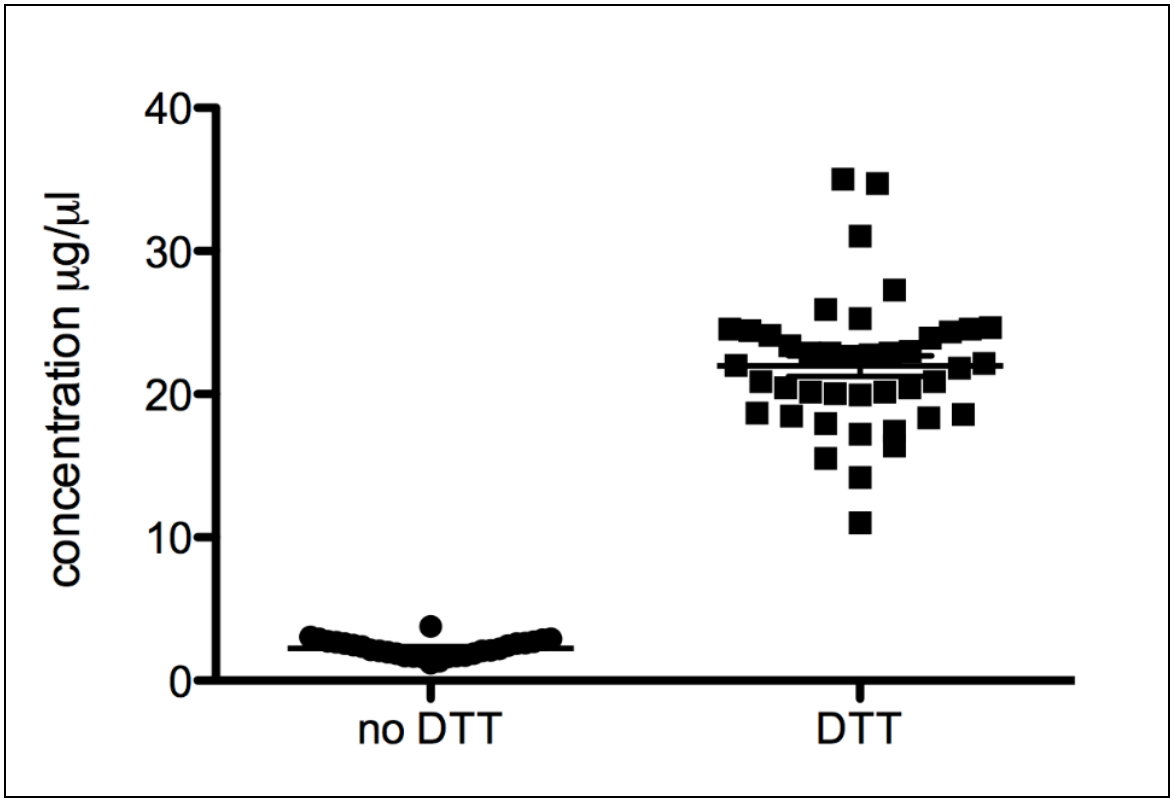


Figure 2.7 Graph showing the distribution of the concentration of proteins extracted using lysis buffers without (buffer A) and with (buffer B) DTT.

2.4 Bradford protein assay

The Bradford protein assay was carried out after extraction of proteins from the heart homogenates to determine the concentration of the protein in the samples. This allows for an equal amount of protein loading with the various molecular assays. The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye, and gives a relatively stable colorimetric response compared to other methods.

The dye solution was prepared by dissolving 50 mg of Coomassie Blue G250 in 50 ml of methanol. The solution is then added to 100 ml of 85% hydrophosphoric acid, and further diluted with 500 ml of distilled water. The solution was then filtered to remove any precipitates. After filtration, another 350 ml of distilled water was added to the final solution. This solution was stored in a dark bottle at 4°C as it remains indefinitely stable in this condition.

Protein standards were prepared using bovine serum albumin (BSA). Nine standards containing 0, 1, 2, 4, 6, 8, 10, 12 and 15 μg/ml of BSA were made. One ml of dye solution was added to the standards and samples to be assayed and mixed with the whirlmixer. The solution was allowed to equilibrate for a few minutes before recording the absorbance at 595 nm using a spectrophotometer. The absorbance of the protein standards was used to plot a standard curve and the standard curve used to determine the concentration of the samples (Fig 2.8).

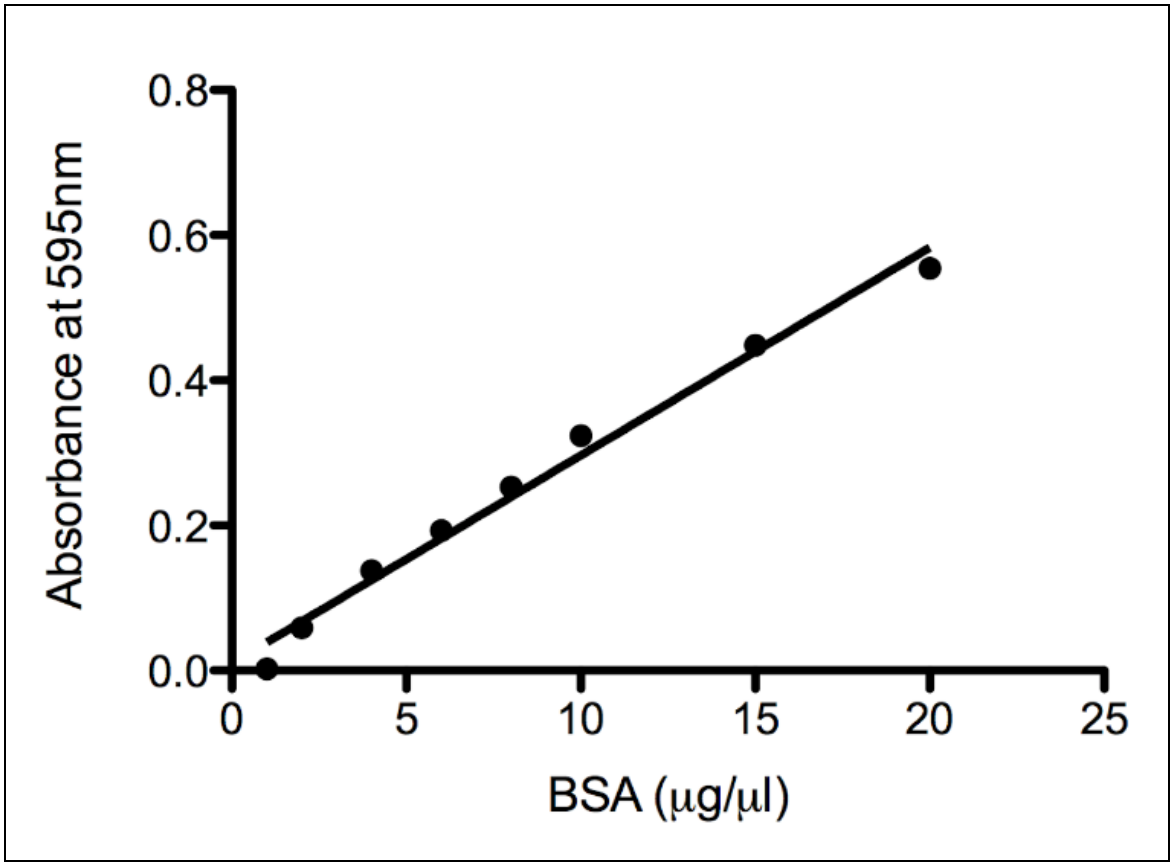


Figure 2.8 An example of a standard curve obtained with Bradford protein assay.

2.5 Protein immunoblot (Western blot)

Western blot is an analytical technique that is widely used to detect specific proteins in a given tissue sample. It is based on the principles of electrophoresis to separate proteins by their molecular weight. The proteins are then transferred to a membrane to be probed using antibodies specific to the target proteins.

2.5.1 Preparation of samples

1. The sample of tissue homogenate was mixed in sample buffer made up with 40% v/v glycerol, 8% w/v sodium dodecyl sulphate (SDS), 0.25 mM Tris, 10% v/v 2-mercaptoethanol and 8% w/v bromophenol blue.
2. 4X sample buffer was used to minimise dilution of the samples. 120 µl of tissue homogenate was added to 40 µl 4X sample buffer.
3. The tissue homogenate in the sample buffer was boiled at 100°C for 5 minutes. The boiling, together with the SDS in the sample buffer will denature and reduce the proteins. The boiled samples were then centrifuged in 4°C at 10,000 rpm for 5 minutes.
4. 50 µg of the proteins (as determined by Bradford assay) was loaded onto the wells.

2.5.2 Electrophoresis and transfer of proteins

1. 8% acrylamide gels were used as the molecular weight of proMMP2 is 72 kDa. Activated and proteolytically-cleaved MMP2 has a molecular weight of 62 kDa. Polyacrylamide gels, which consists of a lower resolving gel (8% acrylamide gel) and an upper stacking gel (6% acrylamide gel) were set between glass plates, separated by 1 mm spacers. The compositions of the gels are listed in Table 2.1. Samples were loaded into wells created by the insertion of a plastic toothed comb (10 wells) into the stacking gels during preparation of the gels.

Table 2.1 Composition of stacking and resolving gels

Resolving gel	Stacking gel
0.375 mM Tris pH 8.8	125 mM Tris pH 6.8
0.1% SDS	0.1% SDS
8% acrylamide	6% acrylamide
0.1% tetramethylethylenediamine (TEMED)	0.1% TEMED
0.15% ammonium persulphate (APS)	0.15% APS

2. A standard running buffer of 1X Tris-glycine was used.
3. Separation of the proteins was carried out using Mini Protean III gel electrophoresis system (Bio-Rad, UK) at 200V for about 30-45 min, or until the dye over-runs the gel at the bottom.

4. The proteins were transferred using electroblotting to expose it on a thin surface layer for detection. A semi-dry transfer system (Bio-Rad, UK) was used.
5. Polyvinylidene difluoride membrane (PVDF) (Hybond P; GE Healthcare, Amersham, UK) was used because of its non-specific protein-binding properties (via hydrophobic and charged interactions between proteins and membrane).
6. The PVDF membrane was cut to size and pre-soaked in methanol prior to use.
7. The PVDF membrane was sandwiched with the resolving gel between pre-soaked (in transfer buffer) Whatman electrode papers.
8. The transfer buffer was made up of 25 mM Tris, 150 mM glycine and 20% methanol. SDS was not used in the buffer as the molecular weight of MMP2 is <100.
9. The sandwich of paper/PVDF/gel/paper was then placed between the electrodes, with the membrane closest to the positive electrode and the gel closest to the negative electrode. Care was taken to ensure the system was well-lubricated with transfer buffer and free of air bubbles to increase the effectiveness of the transfer.
10. The set up is then run at 20V for 120 min.

2.5.3 Blocking and incubation with antibodies

1. Following transfer, the membrane was blocked to prevent non-specific binding of proteins in order to reduce background signal.
2. The membrane was blocked using 5% skimmed milk made up in Tris Buffer Saline Tween 20 (TBST). It was left on an agitator at room temperature for 1 hour.
3. Incubation with primary antibody was then carried out overnight at 4°C on an agitator. The primary antibody (rabbit polyclonal H-76, Santa Cruz) was diluted 1:200 in TBST.
4. The following day, the membrane was washed 6 x 5 min in TBST at room temperature on an agitator.
5. The secondary antibody (donkey anti-rabbit, Santa Cruz) was then incubated for 2 hours at room temperature on an agitator. The dilution used was 1:5,000 in TBST.
6. After 2 hours, the membrane was again washed 6 x 5 min in TBST.

2.5.4 Detection

1. As the antibody used is horseradish peroxidase (HRP)-conjugated, enhanced chemiluminescence (ECL) detection kit is used. The reagents were mixed and allowed to equilibrate with the membrane for 2 min at room temperature.
2. The X-ray films were then developed manually in a dark room with variable exposure times.

2.6 Substrate (gelatin) zymography

The most widely used and conventional method to assess MMP activity is substrate zymography. This is an electrophoretic technique based on SDS-PAGE, where the substrate is co-polymerised with the polyacrylamide gel. Hence, the detection of MMP activity is based on molecular weight separation. It is relatively cheap, sensitive, widely used (hence allowing comparison with other studies) and is a good screening tool.

Sample preparation and tissue extraction is a very critical step in substrate zymography [86, 87]. This is especially true in tissues; the amount of MMPs in tissue is about 50-fold less than that present in culture media, and difficult to extract due to tight binding of MMPs to extracellular matrix. Disruption of the tissues during the extraction process may cause binding of MMPs to its respective inhibitors, which in an intact tissue are localised in distinct compartments. This could potentially lead to inactivation of MMPs. It is important to exclude reducing agents such as EDTA and zinc-chelators from the homogenisation buffer [86]. Additionally, the samples should not be boiled as boiling could activate the enzymes.

2.6.1 Ingredients

The composition of the gels and buffers used in zymography are shown in Tables 2.2, 2.3 and 2.4.

Table 2.2 Composition of gelatin and stacking gel

0.1% Gelatin Gel	Stacking gel
8% acrylamide	6% acrylamide
0.1% Gelatin	water
0.375 mM Tris pH 8.8	125 mM Tris pH 6.8
0.1% SDS	0.1% SDS
0.15% APS	0.15% APS
0.1% TEMED	0.1% TEMED

Table 2.3 Composition of non-reducing loading, renaturation and developing buffers

Non-reducing Loading buffer	Renaturation buffer	Developing buffer
0.25 mM Tris pH 6.8	2.5 M NaCl	1 M Tris pH 7.4
40% v/v glycerol	Triton X-100	2.5 M NaCl
8% w/v SDS	water	1 M $CaCl_2$
8% w/v bromophenol blue		10 mM $ZnCl_2$
water		water

Table 2.4 Composition of stain and destain solutions

Stain solution	Destain solution
25% v/v Methanol	25% v/v Methanol
7% v/v Acetic acid	7% v/v Acetic acid
2.5% w/v Coomassie Blue R250	water
water	

2.6.2 Gel preparation

1. All apparatus was washed and cleaned with 70% ethanol.
2. The ingredients for the running gels (8% gel) were mixed together in a 50 ml Falcon tube. It was mixed together gently to ensure the least amount of air was mixed in as oxygen inhibits the polymerisation process.
3. The running gel was then pipetted into the western blot glass plates, and usually takes about 30 min to set. A small amount of butanol was applied to the top of the gel to ensure no air bubble was trapped. When the gel set, the butanol was washed off with distilled water.
4. The ingredients for stacking gel were mixed together and applied carefully to the top of the running gel. The comb was carefully and quickly slid on top of the gel, ensuring no air bubbles were trapped in the comb.

2.6.3 Electrophoresis and incubation with developing buffer

1. The running buffer used is cold SDS/Glycine/Tris.
2. Samples or 0.34 ng of MMP2 standard (Calbiochem) was loaded onto the wells.
3. Electrophoresis of the gels was carried out surrounded by ice, with a constant voltage of 90 V. It was allowed to run until the dye over-runs the gel at the bottom; this was generally about 150 min.
4. After electrophoresis, the gel was removed from the plates and stacking gel trimmed off.
5. The gel was rinsed in water for about 10s.
6. The gel was then incubated in renaturation buffer 2x30 min.
7. It was then incubated in developing buffer at 37°C for about 36 hours.
8. The following day, after washing the gels twice in water, it was stained in the staining solution for 2 hours at room temperature on an agitator.
9. Subsequently, it was destained in the destaining solution for 4 hours at room temperature on the agitator.
10. The gel was then scanned using GenSnap software.

2.7 MMP2 activity assay

The hearts were homogenised with β-glycerophosphate buffer as previously described in Section 2.3.

The MMP2 activity assay kit was purchased from GE Healthcare; it is specific for MMP2 and allows a precise quantification of active and pro-MMP2. The range of active MMP2 measured, with a longer incubation period, is 0.19-3.0 ng/ml, with a sensitivity of 190 pg/ml. The assay uses a pro-form of a detection enzyme, which can be activated by captured active MMP2, through a proteolytic event (Fig 2.9).

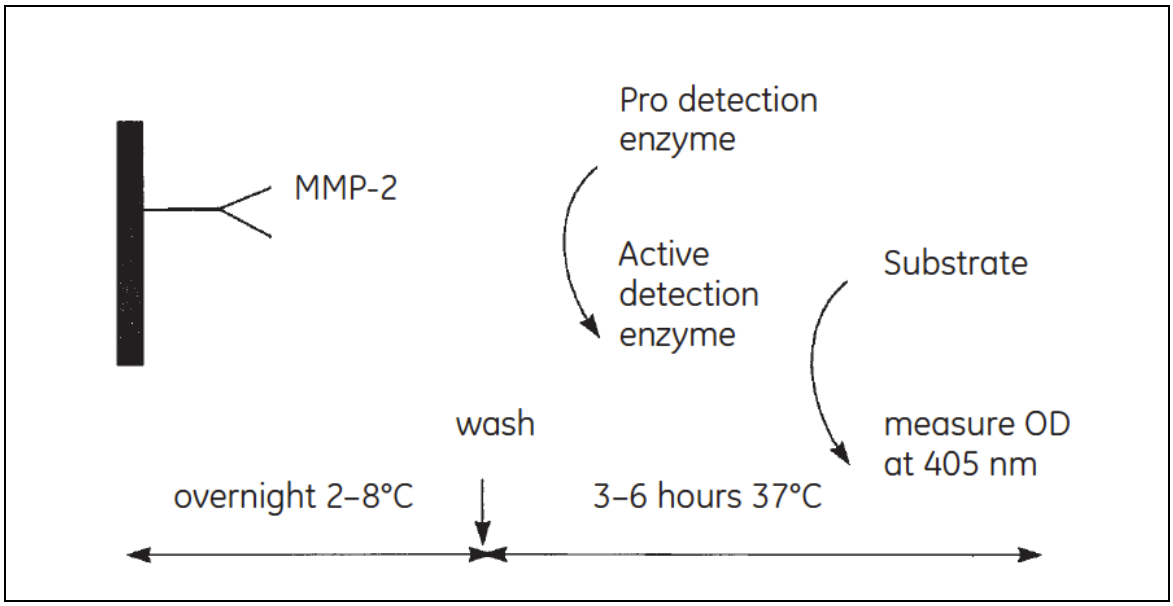


Figure 2.9 Protocol for measuring MMP2 activity

The activated detection enzyme was then measured using a specific peptide substrate. The kit consisted of a 96-well plate coated with anti-MMP2. All assays were carried out in duplicates. The buffers were made up as per instructions in the booklet. A set of 12 working standards, ranging from 0.19 to 12 ng/ml, was prepared. 100 µl of standards and samples were pipetted into the wells. The unknown samples and working standard were incubated overnight at between 2-4°C. Any MMP2 present in the samples was captured in the well. The next day, the wells were washed 4 times before adding the detection enzymes. The optical density (OD) of the plate was read at 405 nm in a microplate spectrophotometer to obtain a t=0 value. The plate was then covered and

incubated at 37°C for 6 hours. Another value for OD was read after the incubation time. A standard curve was constructed, and the concentration of MMP2 in the samples extrapolated from the curve (Fig 2.10).

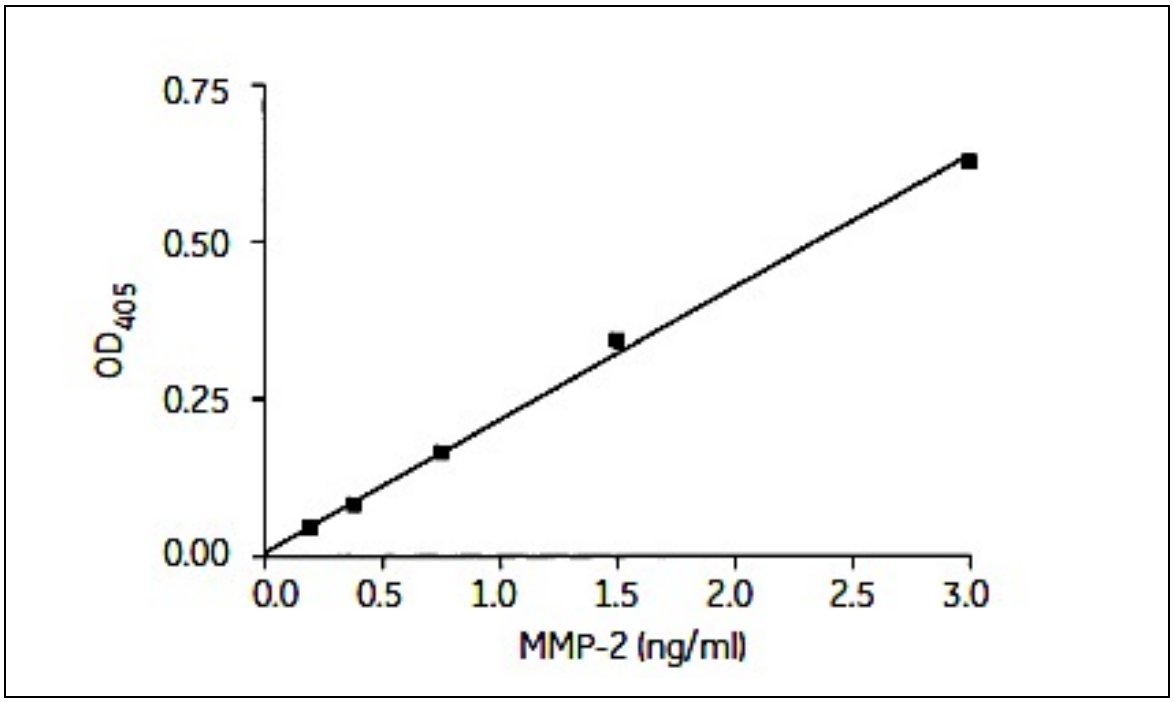


Figure 2.10 Typical standard curve for a 6-hour incubation period

Assays were carried out using old and fresh DMSO. With a preliminary study that was carried out when I first used the assay, the standard curve obtained was very different from what I should expect according to the manual. Following discussion with the manufacturer, the reason for that could be due to DMSO, which was used as a solvent for p-aminophenylmercuric acetate (APMA). APMA was used in the assay to activate MMP2 in the standard. Although a stable compound, when exposed to room air, DMSO will self-dilute to about 66% as it is extremely hygroscopic. Hence, I wanted to ascertain if there was a difference in the assay results when DMSO, that had been opened for more than a year and sitting on the laboratory shelf (old), was used compared to newly bought (fresh) DMSO. Additionally, I also wanted to test the shelf life of the kit. According to the instructions from the manufacturers, once the components were constituted, and if stored according to instructions, i.e buffers at 4°C and reagents at -20°C, the kit should be reusable for a 4-week period. Hence, standard curves were constructed using the same kit at 2 different intervals – in one pair of standard curves, an interval of 10 days was used; subsequently, using another set, the curves were constructed 4 days apart.

Firstly, the comparison of the standard curve using old and fresh DMSO as a solvent for APMA with an expected curve was shown in Fig 2.11i.

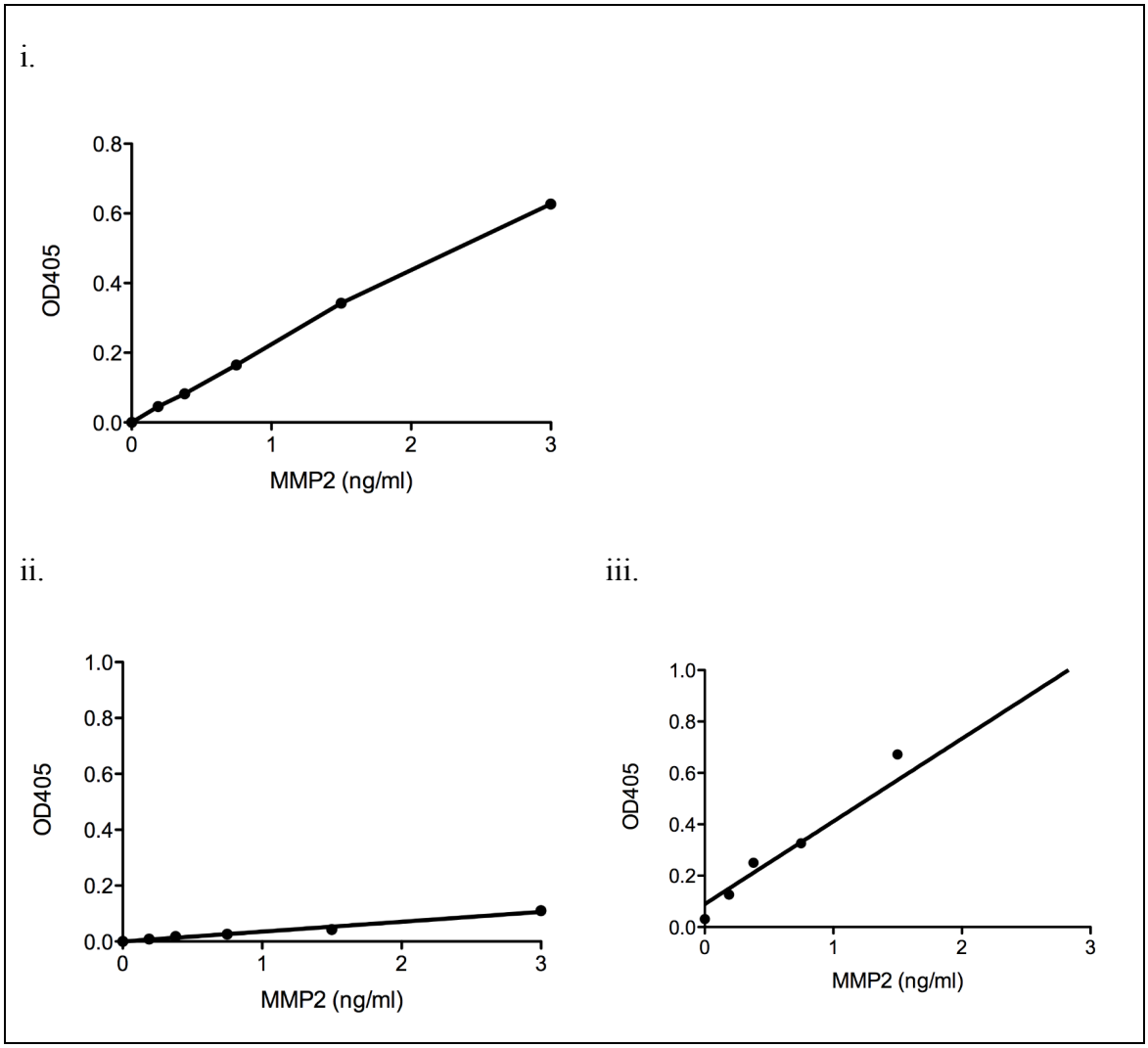


Figure 2.11 Standard curves obtained with MMP2 activity assay.

The standard curves obtained using MMP2 activity assay (i) expected curve reconstructed from the values given in product booklet, (ii) standard curve obtained using old DMSO and (iii) standard curve obtained using fresh DMSO.

As can be seen from the curve obtained, fresh DMSO was crucial to obtain a standard curve that approximated the expected standard curve as given in the product booklet.

The standard curves obtained from the same kit were different when the assay was performed on different days. The curves obtained are shown in Fig 2.12; standard curves obtained 10 days apart showed that the curve approximated to using old DMSO, hence giving a very low standard curve. The effect was less pronounced when the assays were performed only 4 days apart.

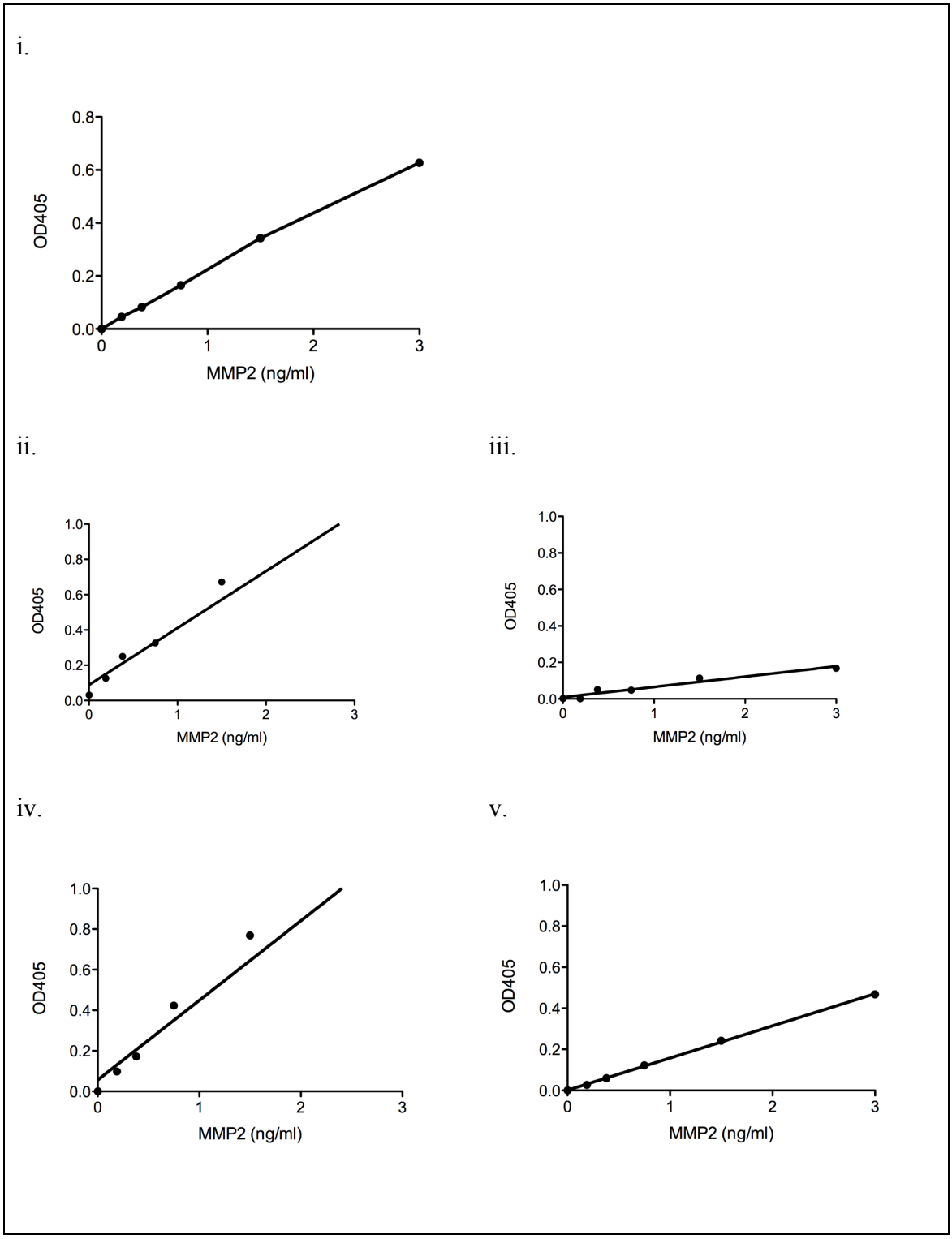


Figure 2.12 Various standard curves obtained using MMP2 activity assay under different conditions.

(i) Expected standard curve, replotted using values given in product booklet. (ii) and (iii) were standard curves obtained from the same kit, but the assay performed 10 days apart. (iv) and (v) were standard curves obtained from the same kit, but assay carried out 4 days apart.

Subsequently, I compared the standard curves obtained using fresh DMSO, from two separate MMP2 activity assay kits that were newly opened, arbitrarily labelled Assay 1 and Assay 2. Both assays were carried out with strict adherence to the conditions instructed by the manufacturer. The slopes of the curves obtained were compared using linear regression and showed significant (p=0.001) differences (Fig 2.13).

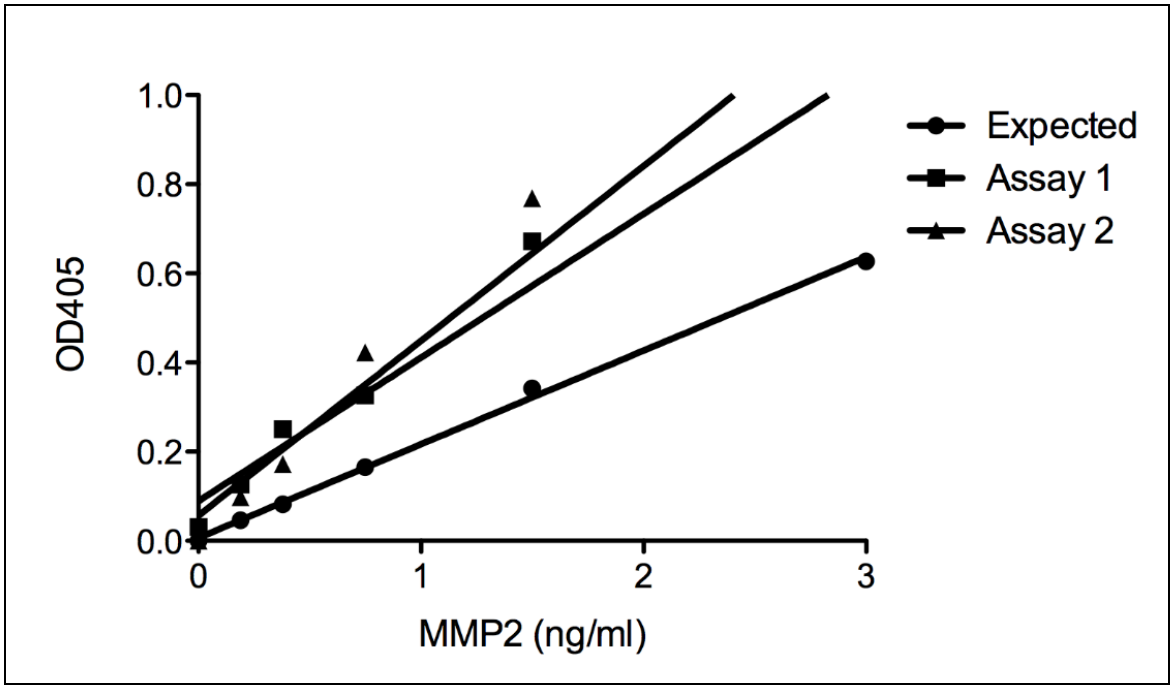


Figure 2.13 Linear regression of standard curves obtained using 2 different MMP2 activity assay kits. The assays were carried out on different days, with 2 separate kits.

In conclusion, it is very important to use fresh DMSO in this assay and preferably the whole plate should be used at one go, and fresh from the package. Given the variability in the standard curve, the quantitative qualities of the kit should be interpreted with caution. It may be more accurate to draw a conclusion on the trend obtained. Caution should also be exercised in trying to interpret results from different plates (performed at different times) as it may not be conclusive. However, although the MMP2 activity assay has its disadvantages (illustrated here), my opinion is that it is still the better assay; it is readily available in the laboratory, relatively easy to perform, and measures directly activity of MMP2s more accurately as the assay is independent of the molecular weights of MMP2.

2.8 Statistical analysis

Statistical analyses were carried out using Prism v5.0f software (GraphPad, USA). All values were expressed as mean±SEM. Comparisons of means of data sets between 2 groups were carried out using unpaired or paired t-test as appropriate. Comparisons of means of multiple data sets were carried out using one-way analysis of variance (ANOVA) with appropriate post-hoc tests for multiple comparisons. Specific statistical analysis is described in the Results chapters as appropriate.

3 PRELIMINARY STUDIES TO DETERMINE THE EXPERIMENTAL DESIGN

3.1 Introduction

The aims and objectives in this study, as mentioned in Chapter 1, section 1.6, were to study the role of MMP2 and mechanical dysfunction in the infarcted heart when subsequently subjected to an additional global ischaemic burden. This mimics closely the group of patients presenting with ACS and undergoing CABG within a short interval from their ACS.

In the past, experimental surgical occlusion of the coronary circulation was popular in dogs to study the consequences of myocardial infarction, due to the similarities in the coronary circulation between human and dogs. Although dogs are convenient animals to perform thoracotomy on, unfortunately, because of their collateral circulation, they are an unreliable animal to use to achieve consistent infarct areas or survival of the animal post-surgery [83, 88]. The resultant infarct size may be minimal due to the collateral circulation or the dogs will not survive post-ligation as a consequence of a large infarct (Fig 3.1). Hence, this caused difficulty in reproducing the results and failure to replicate the laboratory findings in clinical trials. Attention was then turned to other animals, especially small animals, in an effort to either develop a more reliable model or have larger study numbers to achieve statistical significance. Rats were shown to be a good small animal in this respect, as they are logistically manageable in larger numbers. They have consistent anatomic patterns in their coronary circulation, with minimal collateral circulation [82]. This allowed researchers to achieve a consistent ligation result – enabling researchers to produce consistently and sufficiently large infarcted areas, which are compatible with survival. The end results are more reliable due to the larger number of study subjects. Hence, rats are an established ischaemia-reperfusion injury model [89].

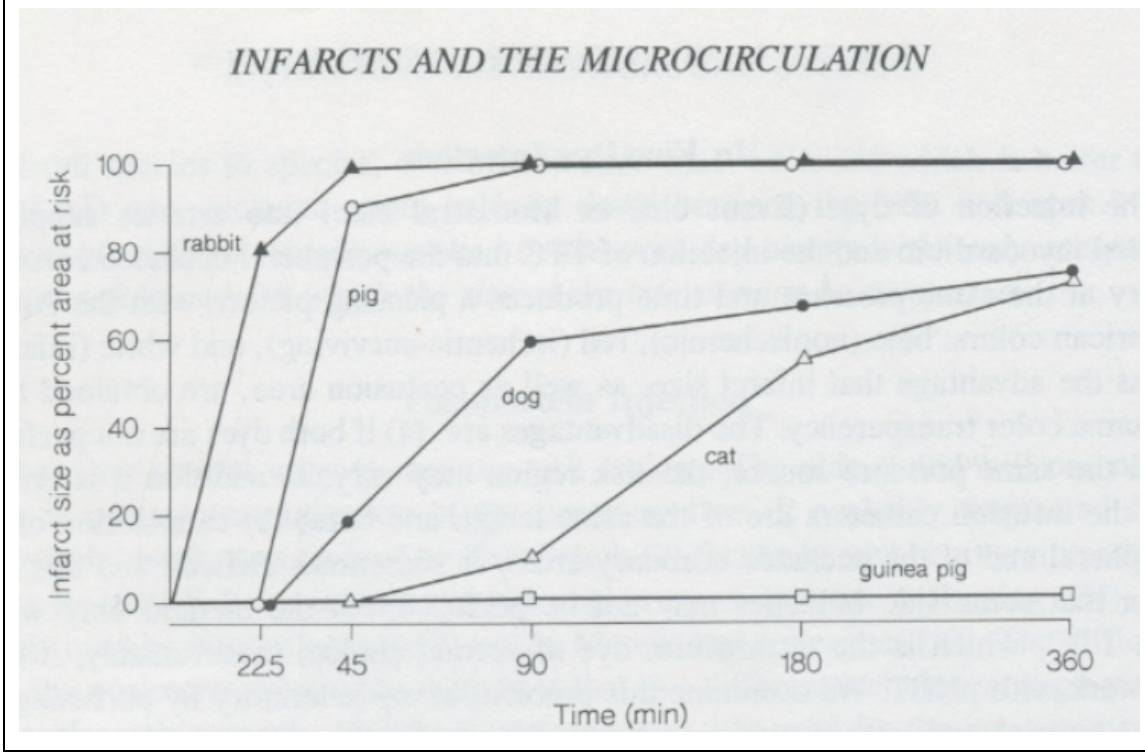


Figure 3.1 Species differences in collateral flow and the rate of development of infarction. Infarct size, expressed as a percent of area at risk in relation to the duration of elapsed ischaemia (followed by reperfusion) in rabbit, pig, dog, rat and guinea pig. The rat (not shown) follows the identical profile to the rabbit. [88]

There will always be inherent limitations when applying laboratory animal data to patients in the clinical setting. Although minimal collateral circulation in rats is an advantage in an experimental set-up, it is also a factor that clearly distinguishes it from patients; patients with acute coronary syndrome usually develop gradual occlusion of the coronary vessels, hence allowing time for collaterals to develop. With this in mind, the rat model of in vivo coronary artery ligation may not be the ideal model in studies looking at ways of minimising infarct size [90]. However, the aims in this study was to look at the effects of an initial infarction on MMP2 activation in the heart and its subsequent action on the heart during elective global ischaemia as would occur during cardiac surgery. Hence, surgical LAD occlusion is a suitable model to achieve these aims as long as consistent infarct sizes are achieved in the animals. Initially, studies were conducted to determine whether my technique induced reproducible and consistent area at risk of the myocardium following surgical ligation, which was at a sufficient size that was compatible with the survival of the animal beyond 24 hours of surgery.

Subsequently, the final infarct size measurement in the isolated hearts at the end of the Langendorff perfusion protocol (when the hearts were not required for tissue samples) was carried out. There are various ways to determine infarction in experimental settings. This can be done either through histological changes or biochemical changes such as dehydrogenase reactions [91, 92]. Sufficient ischaemic time is critical for the changes to appear. As the infarction protocol is 7 days, the infarction would have been established and these changes would have occurred by the time the hearts were assessed for infarct size.

The second part of the experimental design utilised ex-vivo isolated Langendorff heart perfusion. The isolated Langendorff heart preparation is an invaluable experimental model in studies of cardiovascular function and has contributed greatly to the understanding of the heart and its metabolism. It is a cheap and reproducible model and being an ex-vivo model, removes the confounding systemic effects [85, 93]. It enables precise control over perfusate composition and the conditions of perfusion and measurements of a variety of indices of tissue function such as contractile function, and biochemical markers of metabolic activity or ischaemia or infarction can be carried out. Rat hearts are the most stable and best characterised for use in this model.

Despite its advantages, the isolated heart preparation also has several limitations. Its ex-vivo state can be a disadvantage, as it removes the systemic interaction with the heart. It also suffers from structural and functional deterioration with time, although this can be limited with good technique and tissue handling. The type of perfusate may also affect the heart. Crystalloid perfusate differs from blood such that, in the absence of haemoglobin, the required pO_2 of the crystalloid perfusate has to be significantly higher than physiological to deliver sufficient oxygen (and this may be damaging), so needs significantly higher coronary flow and lastly, with reduced oncotic pressure, it leads to rapid tissue oedema. It is generally accepted that a 5-10%/hour reduction in contractile function occurs. Hence, it was important to establish the stability of the preparation with normal and infarcted hearts over a pre-determined period of time as a test of experimental technique.

Subsequently, preliminary studies were conducted on normal rat hearts to standardise the ischaemic duration of hearts required for recovery of LVDP to around 40 to 50%.

Establishing this optimal global ischaemic time ensures that there is sufficient capacity in the heart to recover and additionally, any intervention of interest under study, such as addition of any drugs, if beneficial, will improve the recovery of the function of the heart to an extent that it will be detected statistically.

3.2 Methods

3.2.1 Determination of area at risk of myocardium following LAD ligation

The anaesthesia and surgery in 11 animals were carried out as previously described in Chapter 2, Section 2.1. Following placement of the suture, LAD was ligated as described. The jugular vein was then exposed by an incision and blunt dissection in the supraclavicular triangle, with the right side being preferentially used. Between 0.8-1.0 mls of 3% (w/v) Evans Blue was injected slowly into the jugular vein. The dye would enter the non-ischaemic region of the heart, leaving the ischaemic myocardium (risk zone) unstained (Fig 3.2), and would be observed in the first few seconds post-injection in the heart. After about a minute, the entire animal shows signs of Evans Blue staining. Once this happened, the thoracotomy incision was extended to a full clamshell to enable the heart to be excised quickly and easily. It was rapidly placed in cold 4% formaldehyde and stored at 2-4°C overnight.

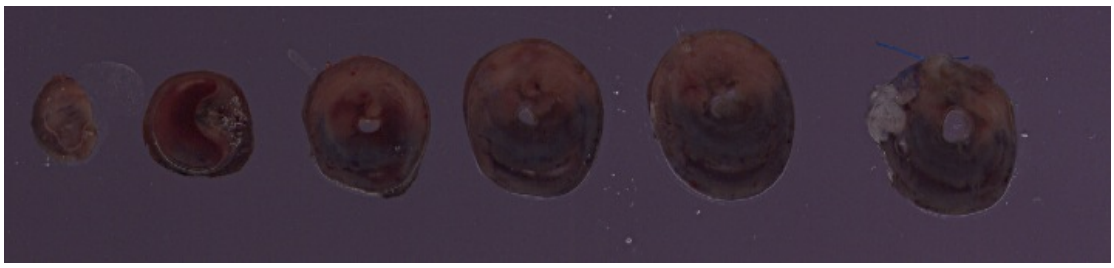


Figure 3.2 Heart slices of 2 mm thickness from apex to base (left to right). Pale (unstained) tissue indicates the area at risk of infarction.

The following day, the heart was sliced using a custom-made multi-blade system, which cut the heart into 2 mm sections (Fig 3.2). The heart sections were scanned immediately, using an Epson Scanner, by placing the sections on the glass scanning plate (with a maximum of 2 hearts at a time) and a Western blot glass plate of 0.75 mm

thickness placed over the top of the sections (Fig 3.3). PBS was then pipetted under the glass plate, at the same time applying slight pressure on the plate to prevent the sections moving around. All the sections were surrounded/immersed in the solution. This was to ensure high resolution with high fidelity images to enable accurate analysis.

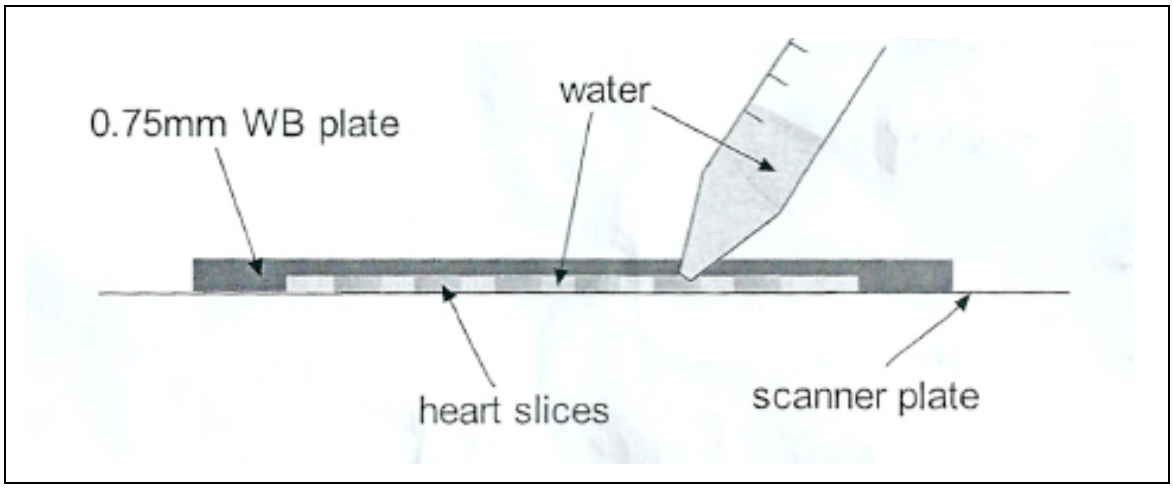


Figure 3.3 Set up of the heart sections for scanning on desktop scanner. (WB – western blot).

The heart slices were scanned at 2400 dpi and images saved as uncompressed TIFF files. The analysis of area at risk was carried out using SigmaScan software. Volumes of the heart were calculated by multiplying the areas by the thickness of the sections (2 mm). The volume of the heart at risk was expressed as the percentage of total ventricular volume of the heart.

3.2.2 Determination of final infarct size following LAD ligation

Myocardial infarction was determined using biochemical changes that occurred within the myocardium with infarction. Triphenyl tetrazolium chloride (TTC) salts have been shown to be a quick and reliable way of assessing infarction [91]. Dehydrogenase enzymes reduce the salt to a dark red formazan pigment in viable tissues, hence necrotic tissue will appear unstained. TTC assessment of viable tissue was found to correlate well with histological examination in well-established infarction [92]. At the end of the isolated Langendorff perfusion protocol, 1% w/v TTC was infused into the heart at 0.5 ml/gm/min. After about 15 minutes of infusion, the hearts were removed from the

cannula and stored at -20°C for 1-2 hours before slicing into 2 mm thickness sections. Infarcted myocardium appeared pale yellow and viable myocardium stained brick red (Fig 3.4). Planimetry of the infarct area was carried out using Image J software. Once again, the volumes of the heart were calculated by multiplying the areas by the thickness of the sections (2 mm) and the infarct size expressed as the percentage of the total ventricular volume.

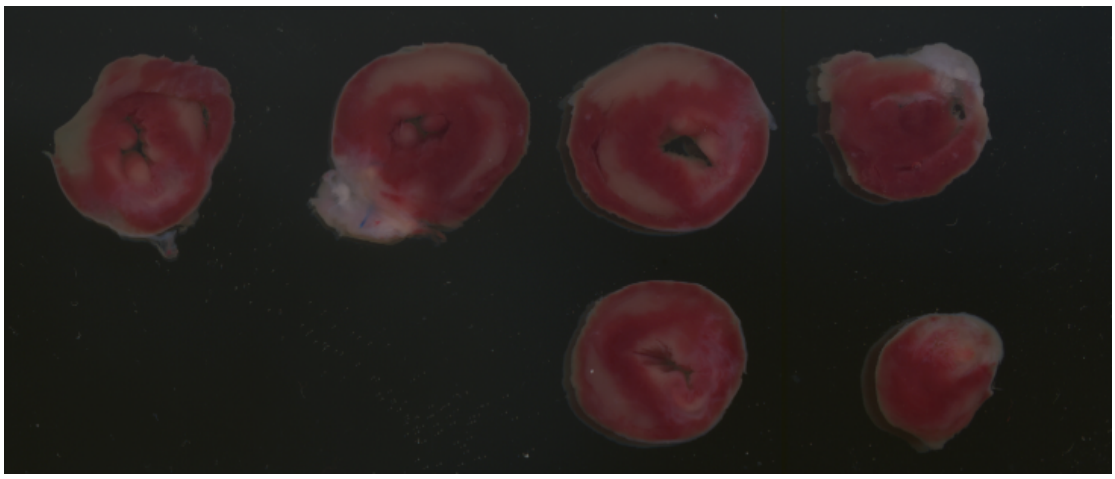


Figure 3.4 Representative heart sections with TTC staining showing infarcted myocardium (pale yellow) and viable myocardium (brick red).

3.2.3 Assessment of stability of isolated Langendorff heart perfusion with normal and infarcted hearts

Normal, adult male Wistar rats (n=3) and adult male Wistar rats that had been subjected to surgical LAD ligation (n=5) 7 days prior to ex-vivo perfusion were used. Surgical ligation of the LAD was as described in Chapter 2, Section 2.1 and the technique of isolated Langendorff perfusion as described in Chapter 2, Section 2.2. The hearts were harvested and perfused with Krebs-Henseleit buffer at a constant pressure of 100 cmH_2O at 37^oC, continuously gassed with a mixture of 95% oxygen: 5% carbon dioxide for 120 min. The HR, LVDP, LVEDP was monitored and acquired continuously, with measurement of coronary flow every 10 min (after the initial 20 min of equilibration); values are expressed as mean±SEM. For infarcted hearts, the exclusion criteria for LVDP was set at a slightly lower value compared to normal hearts. Infarcted hearts that

did not achieve LVDP ≥90 mmHg were excluded from the study. The difference in the changes in each parameter between normal and infarcted hearts was analysed using Student's t-test. A value of $p<0.05$ was considered statistically significant.

3.2.4 Determination of optimal normothermic ischaemic time during isolated Langendorff perfusion protocol in normal hearts

Hearts from normal male Wistar rats (n=3-5 in each groups) were used for isolated heart perfusion as described in Chapter 2, Section 2.2. The hearts were equilibrated with aerobic perfusion for 20 min, before inducing global ischaemia (by clamping the aortic inflow line) for 20, 30 or 40 min, followed by aerobic reperfusion for 60 min (Fig 3.5). The HR, LVDP and LVEDP were monitored and acquired continuously. The coronary flow was measured during baseline aerobic perfusion and every 10 min during the reperfusion period.

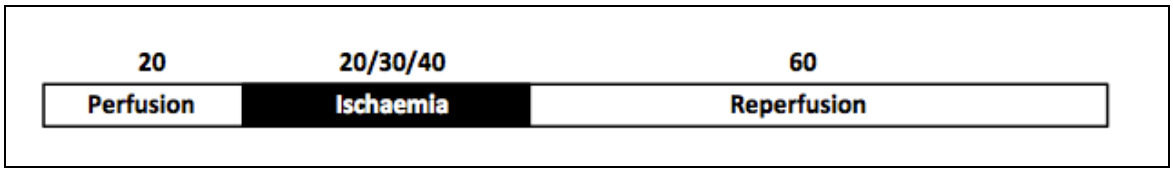


Figure 3.5 Langendorff heart perfusion protocol to determine optimal ischaemic time in normal hearts

Contracture parameters during the ischaemic period were noted. The onset of contracture was defined as a rise of ≥2 mmHg in the LVEDP (Fig 3.6).

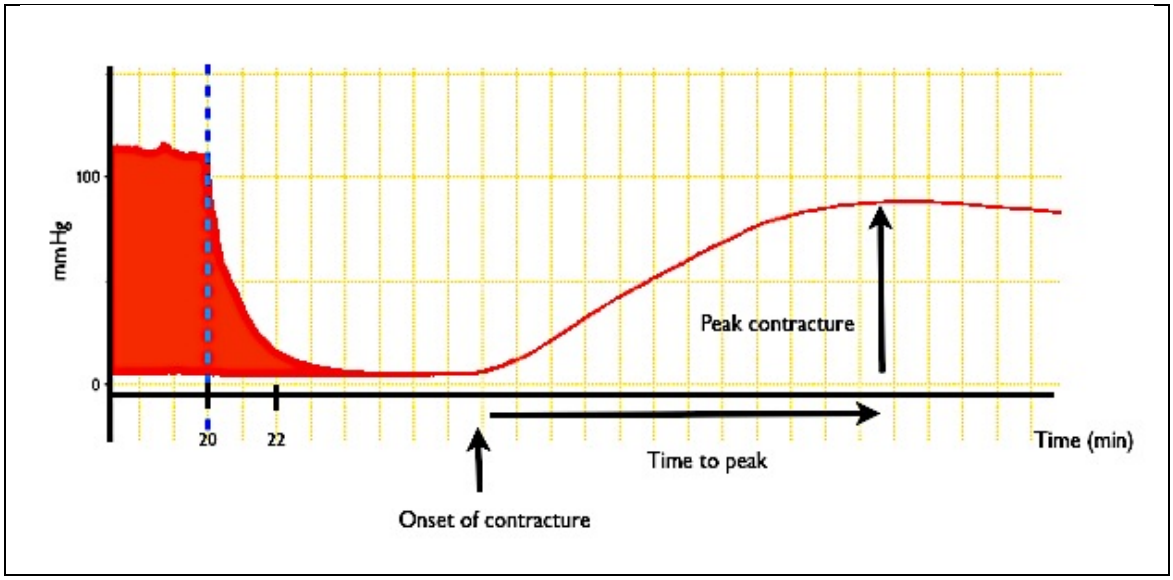


Figure 3.6 Representative LabChart recording during isolated Langendorff perfusion showing continuous acquisition of LVP (LV pressure) over time.

Blue dotted line indicates the time at which the aortic inflow line was clamped, hence inducing global ischaemia. The time taken as the onset of contracture and peak contracture are also marked on the diagram.

3.3 Results

3.3.1 Determination of area at risk of myocardium following LAD ligation

A total of 11 rats were subjected to permanent LAD ligation. In this study, mean area at risk of 49.0± 1.7% following the ligation was achieved. The minimum area was 42.4% and the maximum area was 61.2% with a median of 47.9%. The data showed that the mean and median are very close, with a small range of 95% confidence interval, indicating a tight spread of data (Fig 3.7). Hence, LAD ligation in the rats produced a consistent and reproducible area at risk of myocardium.

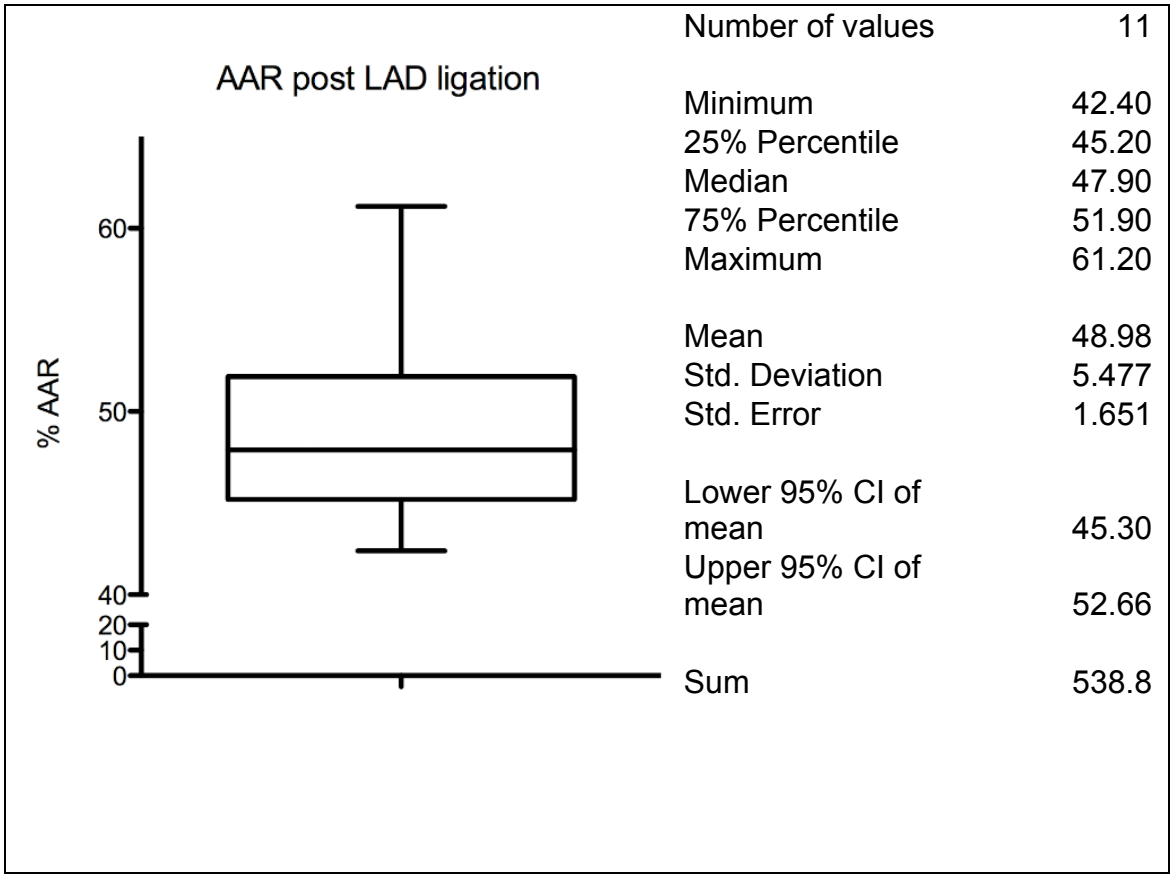
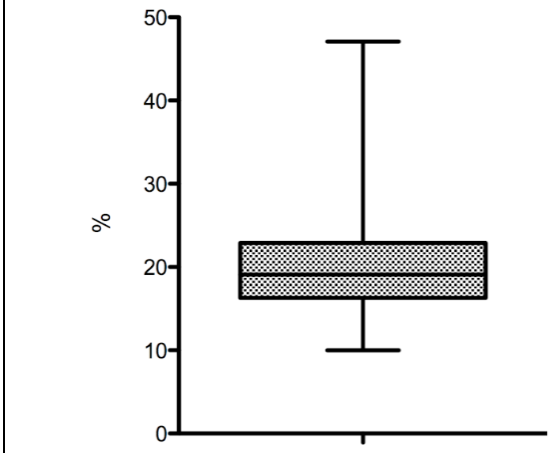


Figure 3.7 Graph showing the distribution of the % area at risk following LAD ligation.

3.3.2 Determination of final infarct size following LAD ligation

A total of 53 infarcted hearts, after various isolated Langendorff perfusion protocols, were included in this analysis. The mean infarct size (expressed as % of ventricular volume) was 20.1±0.9%, with a median of 19.1% (Fig 3.8). The 95% confidence interval lies between 18.4 to 21.8%. This again indicated a tight spread of data, implying that the final infarct size as a result of LAD ligation in these rats were consistent.



Number of values	53
Minimum	10.00
25% Percentile	16.30
Median	19.10
75% Percentile	22.90
Maximum	47.10
Mean	20.10
Std. Deviation	6.269
Std. Error	0.8611
Lower 95% CI of mean	18.37
Upper 95% CI of mean	21.82
Sum	1065

Figure 3.8 Graph showing tight distribution of final infarct size obtained, confirming consistency and reproducibility.

3.3.3 Assessment of stability of isolated Langendorff heart perfusion with normal and infarcted hearts

A total of n=8 infarcted hearts were excluded from the whole study period due to failure to achieve a minimum LVDP of ≥90 mmHg. These hearts that failed to meet the criteria were mostly encountered within the first 4 weeks of the study period, and was probably secondary to my learning curve. The effects of continuous aerobic perfusion in normal and infarcted hearts on LVDP, LVEDP, HR and CF are shown in Fig 3.9. In normal hearts, after 20 min of equilibration with aerobic perfusion, a mean LVDP of 125±12 mmHg was achieved. There was a small dip in the mean LVDP after 60 min of aerobic perfusion, before gradually stabilising again during the second hour of aerobic perfusion, with a further drop towards the end of the perfusion period. After 120 min of continuous aerobic perfusion, the mean LVDP decreased to 96±8 mmHg, which is a total attrition of 22.7±1% in the LVDP compared to baseline. In the infarcted hearts, after 20 min of equilibration, mean LVDP achieved was 128±8 mmHg. Throughout the 120 min continuous aerobic perfusion, there was gradual decrease in mean LVDP.

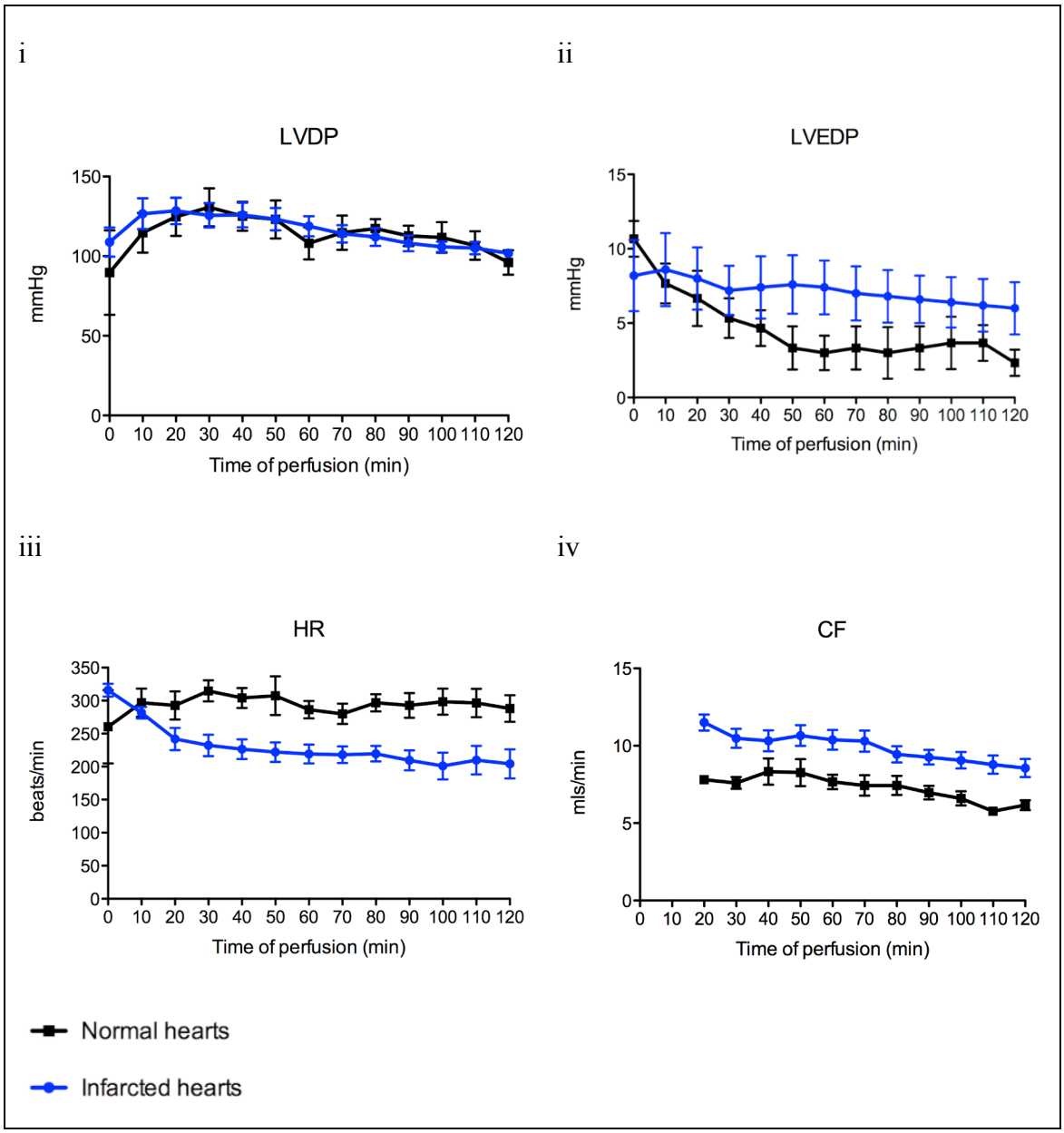


Figure 3.9 Physiological parameters (i) LVDP, (ii) LVEDP, (iii) HR and (iv) CF in normal and infarcted hearts during 120 min of continuous aerobic Langendorff perfusion. n=3 normal hearts and n=5 infarcted hearts.

At the end of the 120 min perfusion, the mean LVDP in infarcted hearts was 102±3, which is a total of 19.5±8% decrease from its baseline value. The % reduction in LVDP in normal and infarcted hearts was not significantly (p=0.59) different (Fig 3.10).

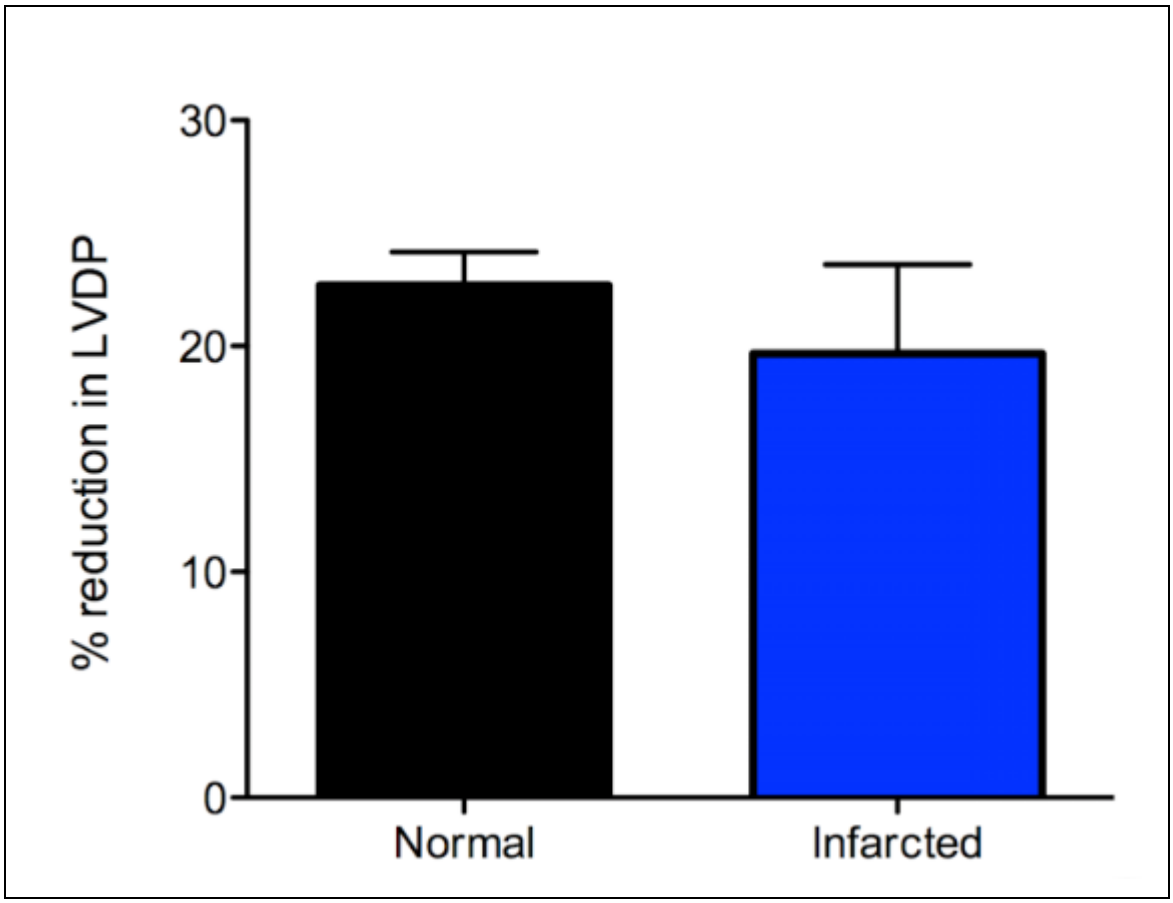


Figure 3.10 % reduction in LVDP in normal and infarcted hearts at the end of 120 min of continuous aerobic isolated Langendorff perfusion compared to after 20 min of equilibration. n=3 normal hearts, n=5 infarcted hearts.

At the beginning of each perfusion protocol, the volume in the balloon was adjusted to achieve LVEDP of between 5 to 10 mmHg. In normal hearts, there was progressive decrease in the LVEDP during the first 60 min of aerobic perfusion, and stabilising for the remaining perfusion period. At the end of the perfusion protocol, there was a 53.7±13% decrease compared to its baseline value. In infarcted hearts, the LVEDP remained fairly constant throughout the perfusion period, with a 25.4±9% drop at the end of perfusion compared to baseline. Again, the change in LVEDP at the end of perfusion compared to baseline values was not significantly ($p=0.12$) different between the 2 groups. The HR in normal hearts was quite stable throughout the perfusion period, whilst the HR in infarcted hearts had a very gradual deterioration throughout the

perfusion period. CF in both groups of hearts again showed gradual deterioration throughout the perfusion period.

Table 3.1 Physiological parameters i.e HR, LVDP, LVEDP and CF (mean values ± SEM) after equilibration with aerobic perfusion for 20 min (baseline) and at the end of 120 min continuous aerobic isolated Langendorff perfusion period, with the % change in the values.

There was no significant (p>0.05) difference in the % change in each parameter between the normal and infarcted hearts at the end of the perfusion period. However, the coronary flow was significantly different between the normal and infarcted hearts at baseline and after 120 min of continuous perfusion.

		Normal hearts (n=3)	Infarcted hearts (n=5)
HR	Baseline	293±21	242±17 (p=0.11)
(beats/min)	End	288±20	204±22 (p=0.04)
	Change (%)	5.7±2	19.5±8 (p=0.26)
LVDP	Baseline	125±12	128±8 (p=0.80)
(mmHg)	End	96±8	102±3 (p=0.42)
	Change (%)	22.7±1	19.7±4 (p=0.59)
LVEDP	Baseline	7±2	8±2 (p=0.68)
(mmHg)	End	2±1	6±2 (p=0.18)
	Change (%)	53.7±13	25.4±9 (p=0.12)
CF	Baseline	7.8±0.2	11.5±0.5 (p=0.002)
(mls/min)	End	6.2±0.3	8.6±0.6 (p=0.03)
	Change (%)	20.8±5	25.7±3 (p=0.39)

3.3.4 Determination of optimal normothermic ischaemic time during isolated Langendorff perfusion protocol in normal hearts

During ischaemia, the time taken for contracture to develop in the hearts subjected to different ischaemic times was not significantly (p=0.56) different (13±2 min in 20 min ischaemia; 11±2 min in 30 min ischaemia; 14±2 min in 40 min ischaemia) (Fig 3.11). The time taken to reach peak contracture was between 16 to 19 min in all 3 groups. The peak contracture was slightly higher in hearts subjected to 40 min of ischaemia at 97±11 mmHg, but again, this was not statistically significant compared to the other 2 groups (79±14 mmHg for 20 min ischaemia and 80±8 for 30 min ischaemia; p=0.47).

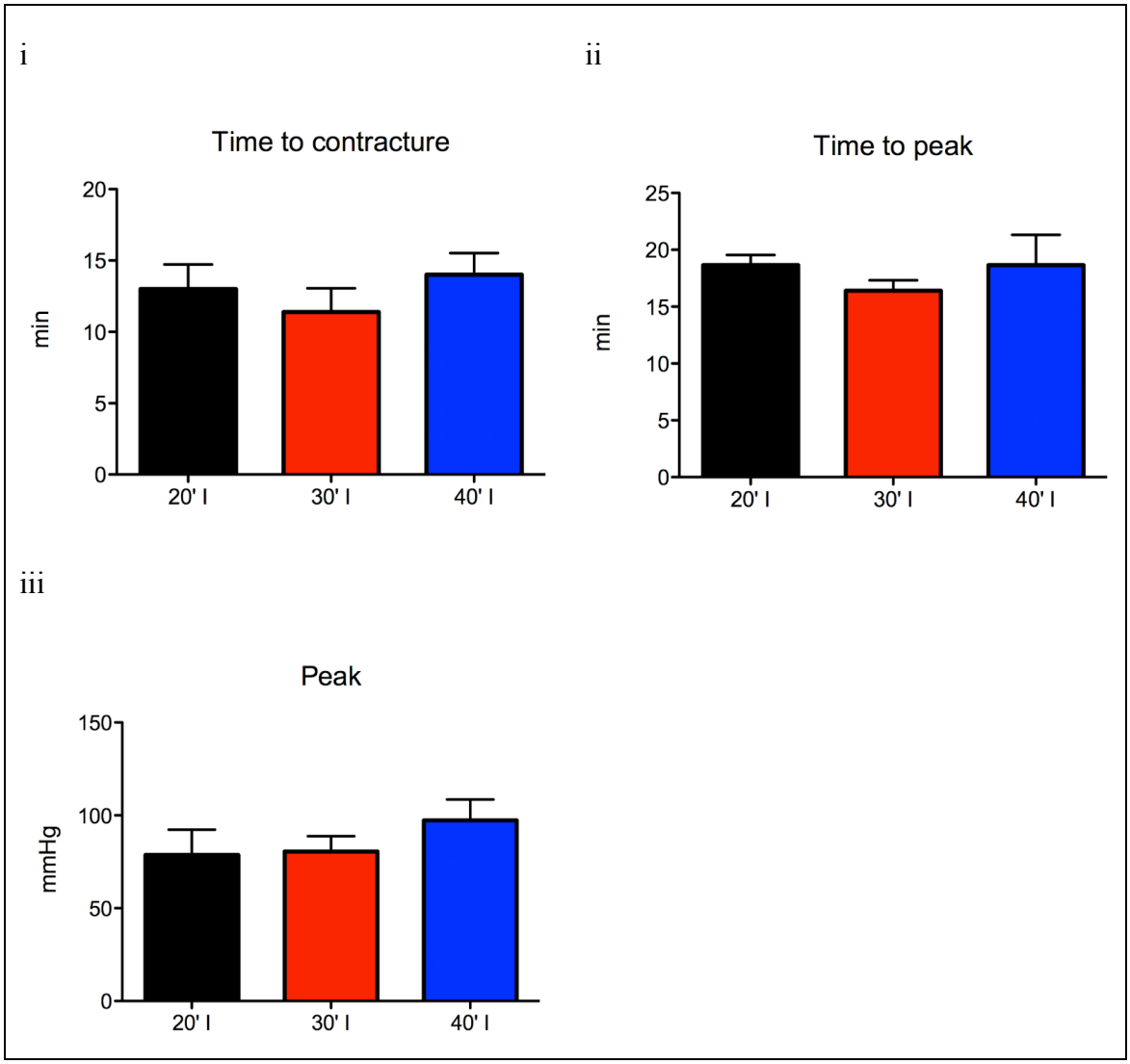


Figure 3.11 Contracture parameters during ischaemia in hearts subjected to 20, 30 and 40 min of global ischaemia. n=3 in 20' I, n=5 in 30' I and n=3 in 40' I.

As expected, increasing durations of ischaemia caused reduced recovery of LVDP; with 20 min of ischaemia, LVDP recovery was much more rapid, even during the early reperfusion period compared to the slow and gradual recovery profile of hearts subjected to 30 or 40 min of ischaemia (Fig 3.12i). The final recovery of LVDP (% pre-ischaemic) at 60 min of reperfusion was 68±13%, 39±5% and 25±6% after 20, 30 or 40 min of global ischaemia respectively (Fig 3.12ii).

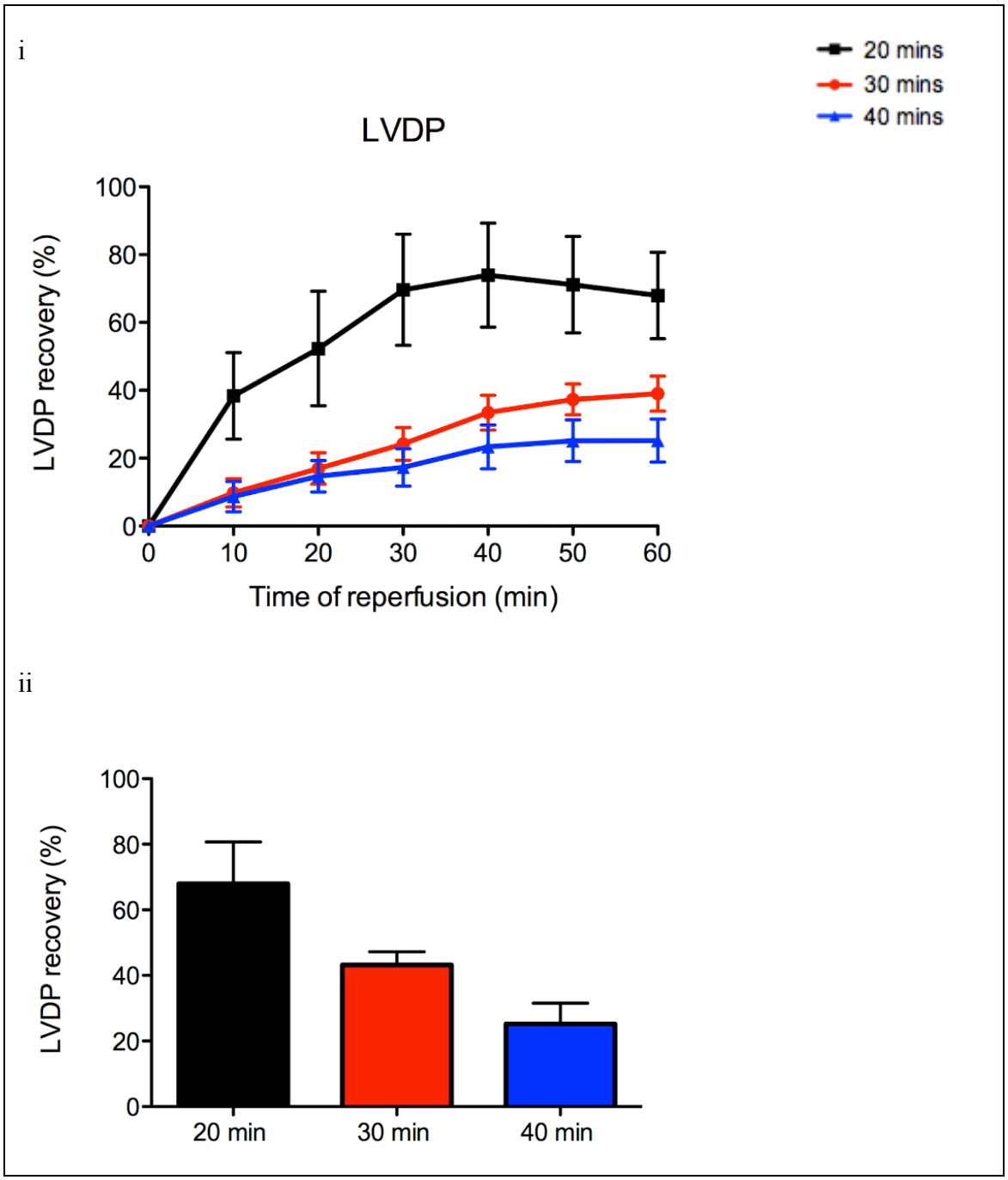


Figure 3.12 Recovery of LVDP during reperfusion after various ischaemic times. (i) Recovery profile during reperfusion (ii) Final recovery of LVDP at 60 min reperfusion. n=3 in 20 min, n=5 in 30 min and n=3 in 40 min ischaemia.

With 20 min of ischaemia, LVEDP continually decreases with reperfusion, but after 30 or 40 min of ischaemia, LVEDP rapidly increases to a peak at 10 min of reperfusion and subsequently declined gradually with reperfusion, but remains more elevated with increasing ischaemic times (Fig 3.13).

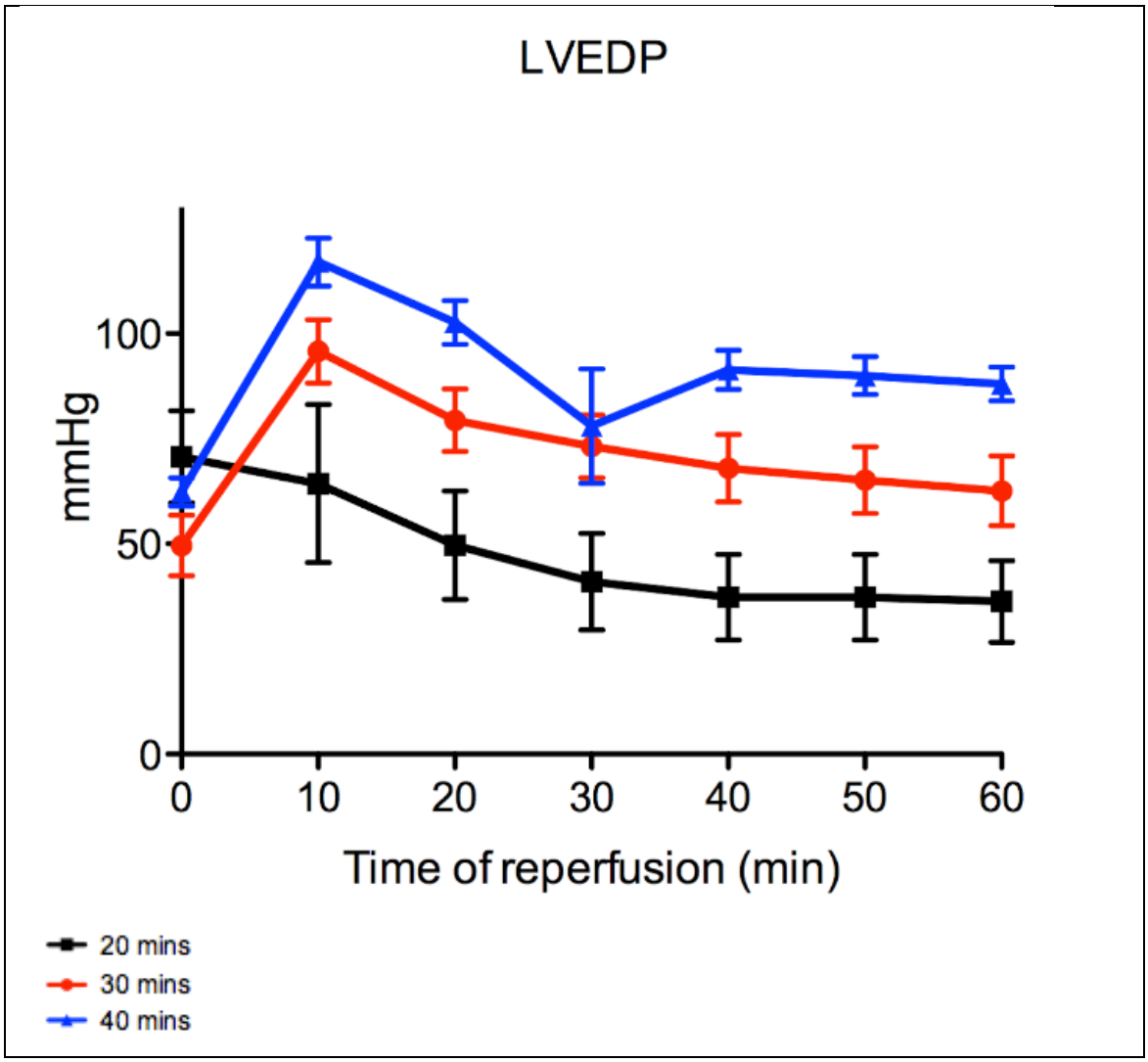


Figure 3.13 LVEDP profile during 60 min of reperfusion after various ischaemic times. n=3 in 20 min, n=5 in 30 min and n=3 in 40 min ischaemia.

The recovery of coronary flow (CF) was also reduced with increasing ischaemic durations. The pattern of recovery was similar in all 3 groups, with rapid recovery within 10 min of reperfusion, followed by gradual decline throughout reperfusion, with a final recovery of 87±4%, 69±9% and 57±3% with increasing ischaemic times respectively (Fig 3.14i). HR recovered well in all 3 groups (Fig 3.14ii).

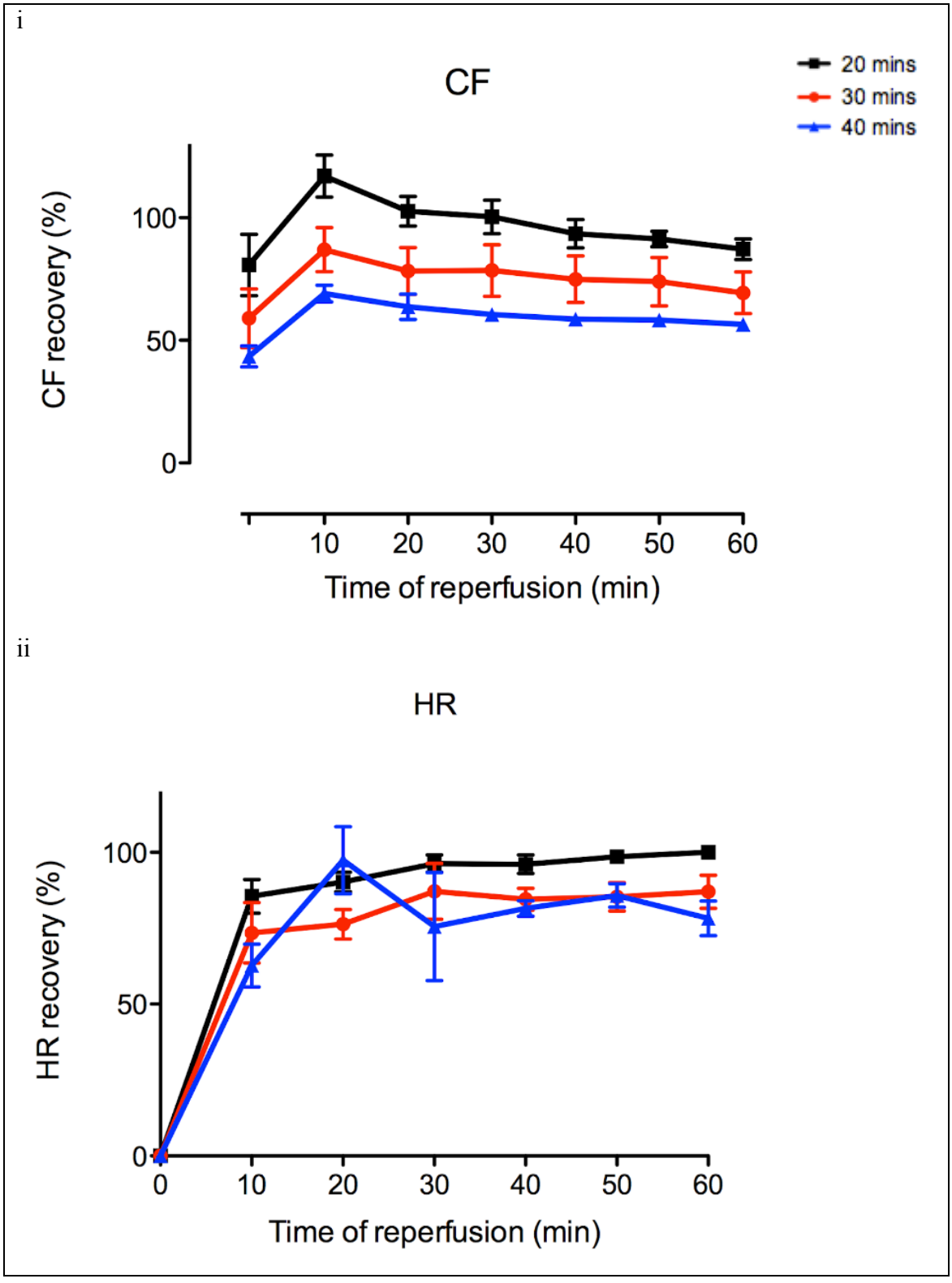


Figure 3.14 Functional recovery of (i) CF and (ii) HR during reperfusion after various ischaemic times. First CF measurement was taken during the first minute of reperfusion. n=3 in 20 min, n=5 in 30 min and n=3 in 40 min ischaemia.

Hence, based on these results, 30 min of global normothermic ischaemia was chosen as the optimal duration for subsequent studies.

3.4 Discussion

In this study, we want to use an animal model that mimics an emerging and clinically relevant scenario, namely, patients who present with ACS needing urgent or emergent CABG surgery. As this is a proof of principle study to determine whether there is a relationship between MMP2 and acute ischaemia-reperfusion in the setting of a previous myocardial infarction, rodents were chosen as a screening animal. In vivo surgical LAD ligation of the animals were used as a means of inducing myocardial infarction. Hence, to standardise the experimental design, it is of utmost importance that the area at risk of infarction of the myocardium following LAD ligation is consistent within animals, even if the ultimate infarct size per se is not the endpoint of cardioprotection in our study. Myocardial infarction is induced in this study to provide an initial injury stimulus to the myocardium to 'prime' or activate MMP2 in the myocardium. Thus, it was a critical balance to produce an area that was sufficiently large to cause significant myocardial infarction, yet not large enough that it was not compatible with survival beyond 7 days. The results obtained in Section 3.3.1 indeed showed that the area at risk of infarction was consistent, implying that the surgical procedure, suture placement and ligation of each animal were consistent. The end result was that the myocardium in each animal was subjected to the same ischaemic stress. The exact procedure carried out was also compatible with survival of the animals for 7 days.

The presence and consistency of ultimate myocardial infarction was also confirmed with assessment of the infarct size. The results in Section 3.3.2 showed that LAD ligation carried out resulted in myocardial infarction and this was again consistent. In the literature, infarct sizes from ligation of LAD range from 4% to about 50% of LV volume [94-99]. Hence, the infarct sizes obtained in this study of around 20% of total ventricular volume are comparable to other laboratories.

Nonetheless, we recognised that the model used here is not without its disadvantages. The rats used were young adult rats, which may not be equivalent to the 60 year-old patients seen in clinical settings. These rats are also healthy rats without any co-morbidity or on any medical therapy for existing diseases. The underlying pathology of

ACS is almost certainly not duplicated exactly in the animals as their coronary arteries are essentially normal.

In the stability studies for isolated Langendorff heart perfusion, all the parameters measured, namely the HR, LVDP, LVEDP and CF remained fairly stable with a slight decrease with time. The attrition of LVDP was about 10%/hour in both normal and infarcted hearts, which is comparable with the general consensus [85, 93]. There was no significant ($p>0.05$) difference between normal and infarcted hearts in terms of the different parameters measured or in the degree of change at the end of the 120 min perfusion protocol compared to after the initial equilibration period. This implied that both the normal and infarcted hearts were stable on this preparation and thus will allow meaningful use of this model to study the effects of global ischaemia on the mechanical function in these 2 groups of hearts. It may have been unexpected that there was no significant difference in LVDP achieved after equilibration with aerobic perfusion between normal or infarcted hearts. I have deliberately excluded infarcted hearts that did not achieve LVDP ≥90 mmHg because it would be difficult to ascertain whether LVDP ≤90 mmHg in the infarcted hearts was due to myocardial infarction or technical problems with the preparation. Unexpectedly, the coronary flow in infarcted hearts after baseline equilibration and at the end of continuous perfusion was significantly higher compared to normal hearts. Remodelling of infarcted hearts 7 days after the initial infarction, leading to increased coronary flow, may account for this difference. Unfortunately, I did not measure the weight of normal or infarcted hearts prior to Langendorff perfusion or at the end of the perfusion protocol.

The function of the heart can be assessed using the relationship between relative intraventricular pressure and volume relationship during cardiac cycle [100]; however pressure-volume relationship in the LV is rather complex. It is not possible to measure volume directly in the LV chamber and hence pressure is usually measured (depending on the model used; mean developed pressure using an intraventricular balloon or direct pressure measurement using a pressure catheter) and used as a surrogate for function. Besides, after infarction, there may be geometrical alteration in the LV chamber, with thinning of infarcted region and compensatory hypertrophy of uninfarcted region of the myocardium. It would have been ideal to be able to carry out haemodynamic studies with pressure volume (PV) catheters to acquire PV loops for each heart. This allows

accurate characterisation and comparison of haemodynamic function in normal and infarcted hearts. However, due to the constraint of time, it was not possible to carry out this part of the investigation even though this technology was available.

Although there was no significant difference between the baseline LVEDP or at the end of the perfusion protocol between normal or infarcted hearts, there was a trend towards a slightly higher LVEDP in the infarcted hearts at the end of the 120 min perfusion. It is important to note that the balloon volume was not adjusted at any time during the perfusion period after the optimal values for LVDP and LVEDP were achieved shortly after cannulation of the isolated hearts i.e at the beginning of the perfusion. A higher volume required to inflate the balloon to achieve adequate LVDP may imply increased wall tension in the LV, hence increasing myocardial oxygen consumption. This model is a rather over-simplification of an extremely complex relationship with multiple assumptions made. After much deliberation and contemplation, we decided to accept the findings at face value; that infarcted hearts could indeed achieve a reasonable and equivalent LVDP to normal hearts when perfused in an isolated heart perfusion preparation, albeit with a slightly higher LVEDP (but not statistically significant). With these preliminary studies, it was important to establish and show that, a consistent area of myocardium at risk of infarction with surgical LAD ligation was induced and that after 7 days, the infarcted hearts were a stable preparation in an ex-vivo isolated Langendorff heart perfusion over a 120-min period. It perhaps correlates rather well with the experience clinically, with the majority of patients undergoing CABG having a good ejection fraction, which is a common clinical index of cardiac function [70, 71]. Patients with ACS, especially in the NSTEMI subgroup, often have good LV, with good ejection fraction, as measured by transthoracic echocardiography. In an unstressed state, an infarcted heart could compensate perfectly well to achieve an adequate cardiac output. However, when stressed further by global ischaemia, such as that imposed by elective cardiac surgery, the heart may not recover as well as a heart not recently stressed by myocardial infarction.

The aim in the final part of this section was to establish the optimal global normothermic ischaemic time in normal hearts. If the ischaemic time is too short, the hearts will recover to a high level and it will be difficult to detect any statistically significant improvement due to pharmacological intervention. On the other hand, if the

ischaemic time is too long, there may be irreversible cellular damage leading to poor recovery, which is not amenable to supposedly beneficial intervention improving recovery. As expected, there was progressive decline in the final recovery of LVDP with increasing duration of global ischaemia. From previous experience in the laboratory, the ischaemic time that results in approximately 40% recovery of LVDP compared to the pre-ischaemic function is usually ideal.

In summary, we have shown that with surgical ligation of the LAD artery, consistent area at risk of infarction and final myocardial infarction were produced in the rats, allowing us to produce rats that resemble patients in actual clinical setting with myocardial infarction. I have also demonstrated that both normal and infarcted hearts were stable under my experimental conditions and techniques over a period of 120 min of continuous aerobic perfusion using the isolated Langendorff heart perfusion preparation. In addition, an ischaemic time of 30 min of global no-flow ischaemia was shown to be the optimal ischaemic duration to examine further interventions aimed at determining the role of additional global ischaemia in previously infarcted hearts. This will be described in the following chapters.

4 PHYSIOLOGICAL EFFECTS OF ADDITIONAL ISCHAEMIA-REPERFUSION ON INFARCTED HEARTS

4.1 Introduction

As mentioned in Chapter 1, there are various studies such as the National Adult Cardiac Surgical Database Report by the Society for Cardiothoracic Surgery of Great Britain and Ireland [70] and the review using California Discharge Data [71] showing that patients with ACS requiring urgent or emergency CABG have an associated higher mortality and morbidity. Recently, diagnostic sensitivity and specificity has improved to the extent that patients can be further classified into different categories within the spectrum of ACS. Emergency CABG (within 48 hours of STEMI) in patients with STEMI carries a much higher mortality, up to 20% in the series published by Hagl et al [66]. In the joint guidelines published by ESC/EACTS, emergency CABG in patients presenting with STEMI is only advocated when primary PCI has failed or is not possible, or when a significant proportion of the myocardium is at risk and surgical revascularisation could be completed before further necrosis occurs [65, 67]. As for urgent CABG, there was a strong inverse relationship between the outcome of surgery and the time that elapsed since STEMI [64, 101]. In other words, CABG should only be considered as the last resort, or only an option if there were other indications, such as mechanical complication in terms of acute rupture of the chordae tendinae or ventricular septum.

The optimal treatment strategy in patients with NSTEMI is not as well-defined as STEMI patients (American Heart Association (AHA)/ESC guidelines) [67, 102]. One of the reasons is that patients presenting with NSTEMI are a very heterogenous group. Current efforts are directed at risk stratification of this cohort of patients to develop a tool to direct management according to risk factors. Risk scores are used to identify the optimal timing of invasive diagnostic tests in the first instance. The other major issue to be resolved is the type of revascularisation, including the role of surgery in the management strategy. There are currently no available randomised controlled trials

(RCTs) to compare different revascularisation strategies in this group of patients. Propensity studies may shed some light but are unlikely to provide the ultimate answer.

The timing of surgery from diagnosis of MI has not been resolved currently. Some studies suggested that the risk of surgery for patients with acute MI was not greater compared to elective CABG [103, 104]. However, the timing of surgery may vary from 7 to 30 days following MI. DeWood and co-workers reported significant improvement in global and regional wall motion of the LV if surgery was performed very early within 6 hours of MI, with a reasonable mortality rate of 2.1% [105]. But, a more recent study by Voisine et al contradicted this finding [106]; in their cohort of patients, peri-operative mortality in patients having CABG within 6 hours of experiencing MI was 19.2%. Interestingly, the increased mortality was not seen in patients under the age of 65 when CABG was performed within 6 hours of MI. However, if the window of 6 hours was missed, the risk of surgery was shown to increase within 48 hours and up to 3 or 7 days of MI [71, 101]. Weiss and co-workers [71] raised the possibility of biological factors such as enhanced systemic inflammatory response being the culprit for the increased risk, as was certainly implicated in a study by Dixon with upregulation of MT1-MMP activity [42]. Alternatively, human or logistical issues (such as an emergency situation with less staff and less controlled environment), may be a contributing factor. Our studies aim to use an animal model simulating the clinical scenario to study the contribution of biological factors such as priming of bioactive molecules from an initial ischaemic insult on the myocardium. Additionally, the effect of timing (3 days vs 7 days post MI) for an elective global ischaemia-reperfusion injury (as would occur during cardiac surgery) imposed on the infarcted myocardium, in terms of the recovery of the myocardium, was also studied using an isolated rat heart perfusion model.

4.2 Methods

4.2.1 Effect of additional global ischaemia-reperfusion on infarcted hearts

The protocols for these studies are shown in Fig 4.1. Four groups (n=5-6 hearts/group) of isolated hearts were perfused using the Langendorff perfusion model as previously described in Chapter 2, Section 2.2. Group (i) was the control group where normal (i.e non-infarcted), adult male hearts were used. Group (ii) consisted of adult male hearts where the LAD was surgically ligated as described in Chapter 2, Section 2.1, to induce myocardial infarction, with the rats allowed to recover for 7 days prior to harvesting the hearts for ex-vivo perfusion. Group (iii) was adult male hearts subjected to LAD ligation as described, with the rats allowed to recover for 3 days prior to harvesting the hearts for ex-vivo perfusion. Group (iv) was adult male rats subjected to sham thoracotomy; the rats were anaesthetised and underwent anterior thoracotomy up to exteriorisation of the hearts (the pericardium was opened and the heart was also exteriorised temporarily) as Group (ii), but suture ligation was not applied to the LAD. The rats were also allowed to recover for 7 days prior to the experiments.

Isolated Langendorff heart perfusion was carried out as described in Chapter 2, Section 2.2. The hearts were equilibrated with aerobic perfusion for 20 min when baseline function parameters were measured, before global ischaemia was induced by clamping the aortic inflow line for 30 min (as defined in studies described in Chapter 3, Section 3.3.4). Global ischaemia was maintained at 37°C. After this period, the hearts were reperfused for a further 60 min with aerobic perfusion (Fig 4.1).

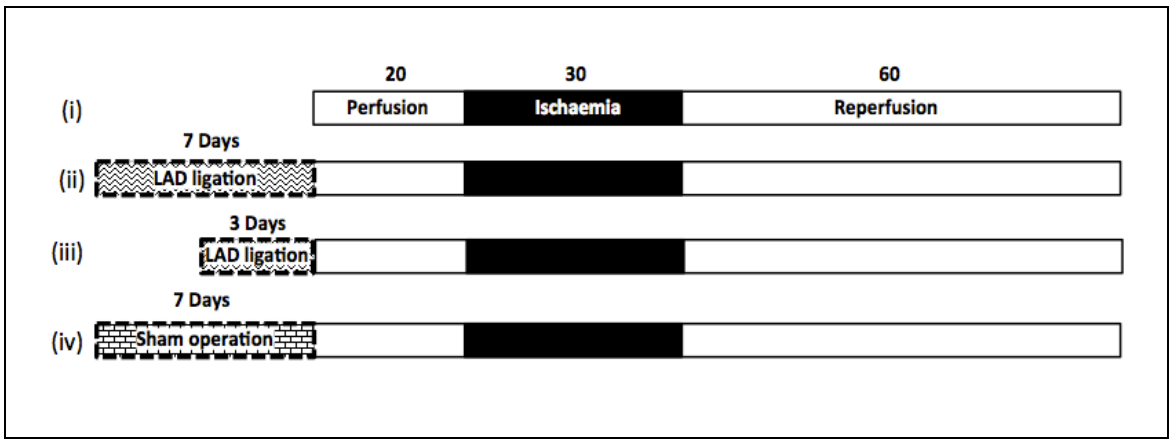


Figure 4.1 Langendorff perfusion protocol for isolated heart perfusion.

The HR, LVSP and LVEDP were monitored and acquired continuously and coronary flow was measured every 10 minutes during the reperfusion period. LVDP was derived from LVSP-LVEDP. The recovery profiles of the groups were compared using the exponential association analysis, which is a nonlinear regression analysis by constructing a best-fit curve and comparing the constants of the best-fit curve between the groups. The final recovery was compared using ANOVA, with post-hoc Dunnett's test for multiple comparisons. A value of $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Contracture parameters during ischaemia

Contracture parameters such as the time taken from ischaemia to develop contracture, the time taken to develop peak contracture and the peak contracture developed were considered. The time taken for hearts in each group to develop contracture (from cessation of aerobic perfusion) were 11±2 minutes, 14±0 minutes, 12±1 minutes and 14±0 minutes in normal, 7-day infarct, 3-day infarct and sham hearts respectively (Fig 4.2i). These were not significantly ($p=0.1$) different. The times taken to reach peak contracture were also not significantly ($p=0.3$) different between the groups, ranging between 16 to 18 min (Fig 4.2ii). The peak contracture developed in the hearts were 86±11 mmHg in normal, 50±8 mmHg in 7-day infarct, 53±4 mmHg in 3-day infarct, and 71±6 mmHg in sham hearts respectively (Fig 4.2iii). The peak in normal hearts was significantly ($p=0.006$) higher compared to either the 7-day infarct or 3-day infarct hearts.

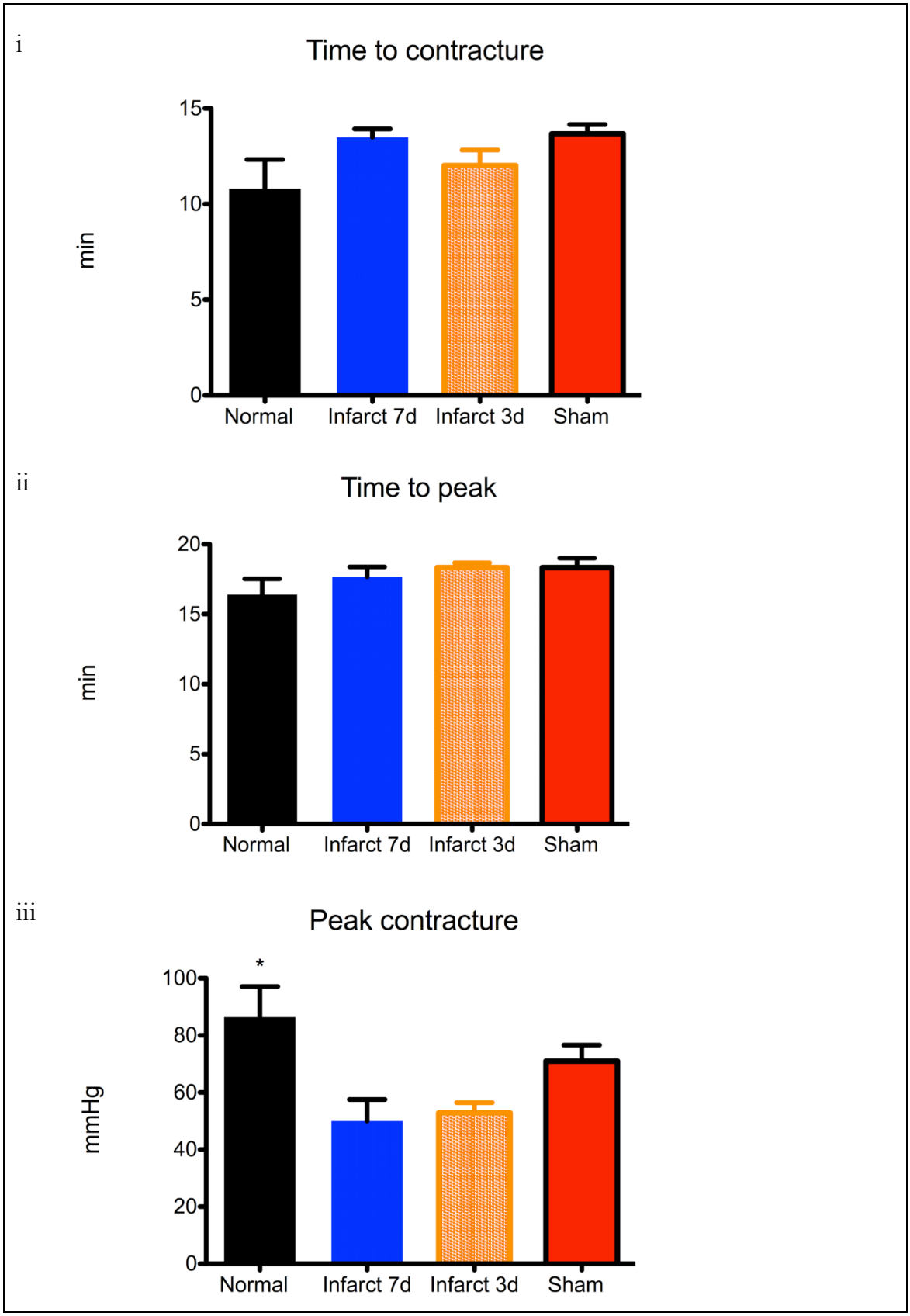


Figure 4.2 Contracture parameters (i) time to contracture, (ii) time to peak contracture and (iii) peak contracture developed during ischaemia; n=5 normal, n=6 infarct 7d, n=6 infarct 3d and n=6 sham. * p=0.006

4.3.2 Recovery during reperfusion

The recovery profiles of LVDP (% pre-ischaemic) of normal, 3-day infarct and sham hearts during the 60 min reperfusion period were very similar. There was slow and gradual recovery throughout the 60 min reperfusion period, reaching a plateau around 50 min of reperfusion (Fig 4.3i). On the other hand, LVDP recovery in the 7-day infarct group was significantly ($p<0.0001$) worse, reaching a lower plateau at 40 min reperfusion. The final LVDP recovery in the 7-day infarct hearts was only 22±2%, which was significantly ($p<0.02$) lower compared to the other groups (39±5% in normal, 32±5% in 3-day infarct and 39±3% in sham hearts) (Fig 4.3ii).

LVEDP in all groups at the end of global ischaemia was higher than baseline, reaching a peak within 10 min of reperfusion, before gradually decreasing throughout reperfusion, although it remained more elevated compared to baseline (Fig 4.4). The LVEDP at the end of reperfusion ranged between 56 – 73 mmHg and was not significantly ($p=0.45$) different between the groups.

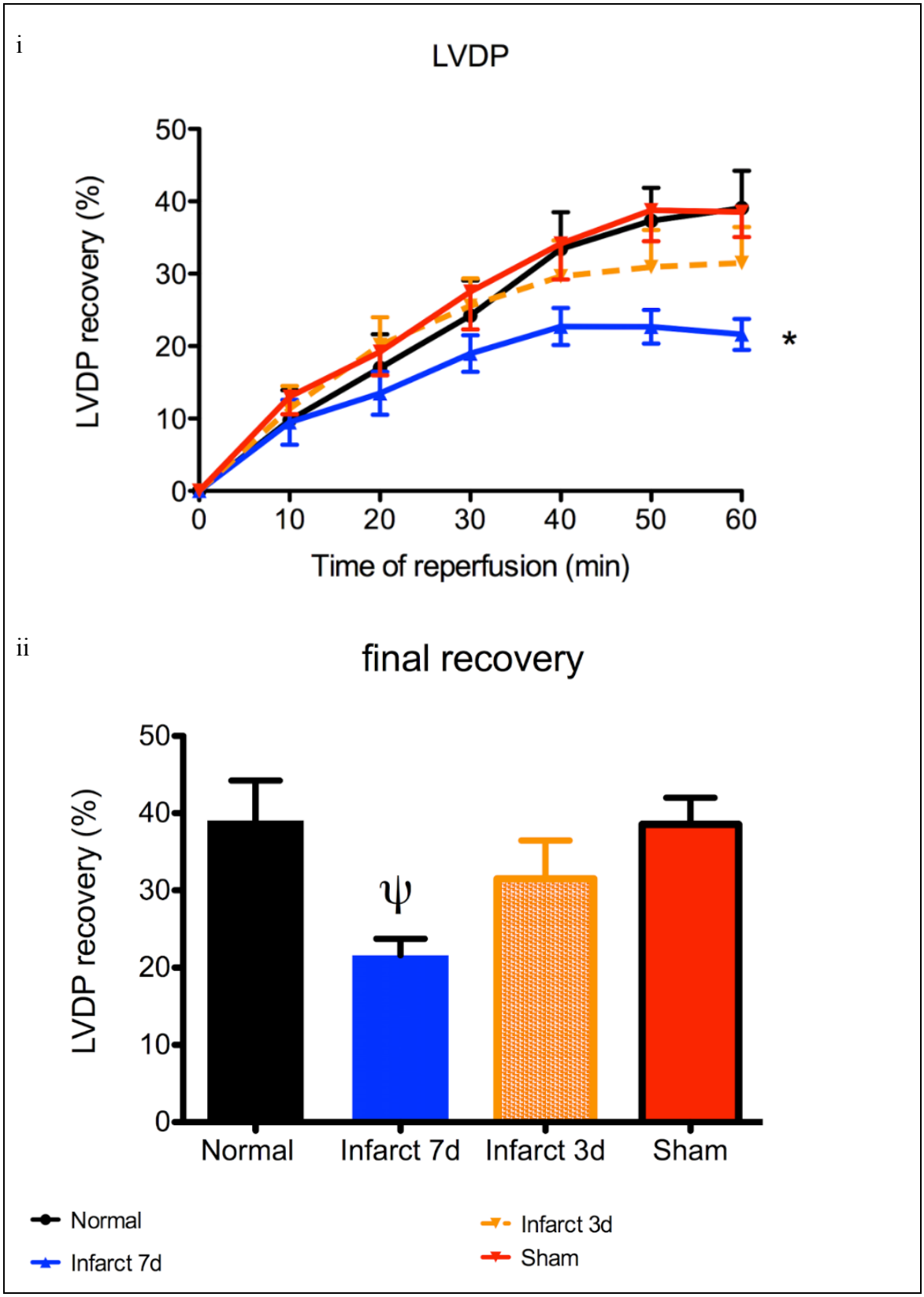


Figure 4.3 Recovery of LVDP (% pre-ischaemic values) in each group during reperfusion after global ischaemia. n=5 normal, n=6 infarct 7d, n=6 infarct 3d, n=6 sham.

(i) LVDP recovery profiles * p<0.0001 (ii) Final LVDP recovery in each group. Ψ p<0.02

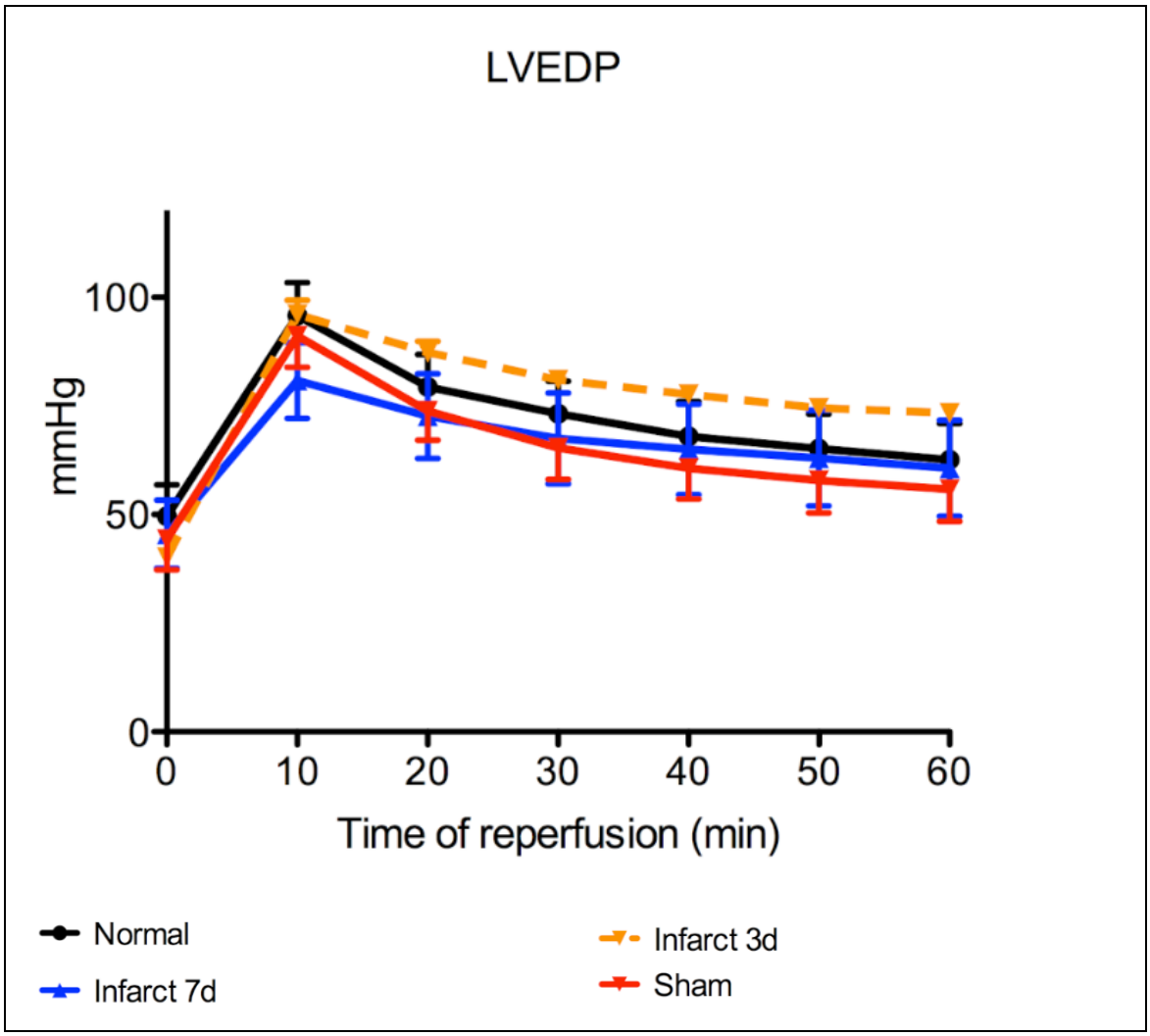


Figure 4.4 Recovery of LVEDP during reperfusion after global ischaemia. n=5 normal, n=6 infarct 7d, n=6 infarct 3d and n=6 sham.

The recovery profiles for HR and CF during the 60 min reperfusion phase are shown in Fig 4.5. The recovery of HR in all groups was very similar. There was rapid recovery to close to baseline (pre-ischaemic) values within 10 minutes of reperfusion. The final recovery (% pre-ischaemic) of HR for normal, 7-day infarct, 3-day infarct and sham hearts were 87±5%, 83±4%, 88±4% and 83±7% respectively. Similar rapid recovery was seen with CF, although it did not recover quite as well as HR. The final recovery of CF showed that the infarcted group tended to have a lower recovery (normal 69±9%, 7-day infarct 56±3%, 3-day infarct 47±1% and sham 65±7%). The final recovery of CF in 3-day infarct hearts was significantly ($p=0.05$) lower compared to normal hearts.

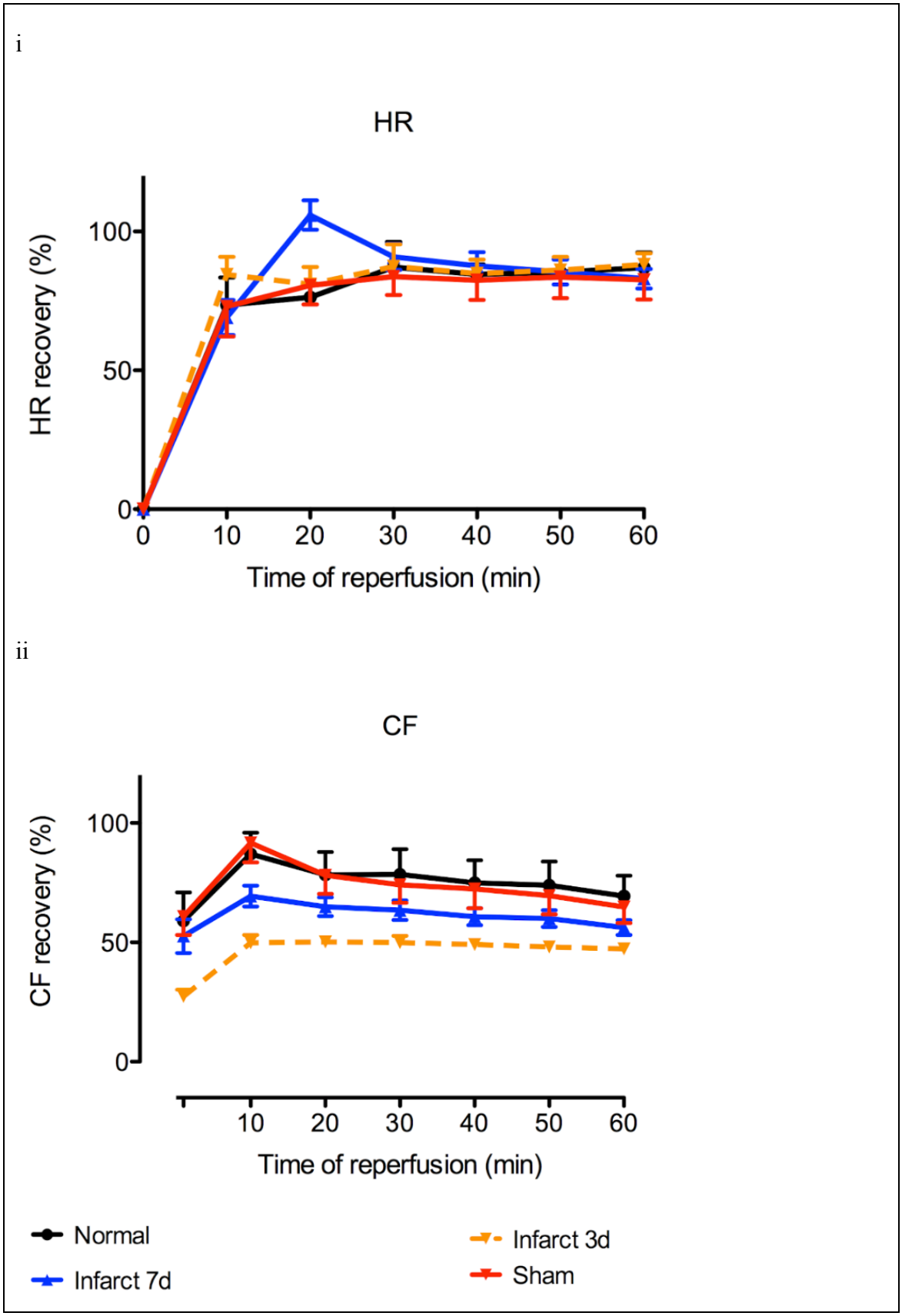


Figure 4.5 Recovery of (i) HR and (ii) CF during reperfusion after global ischaemia. First CF measurement taken during the first minute of reperfusion. n=5 normal, n=6 infarct 7d, n=6 infarct 3d and n=6 sham.

4.4 Discussion

The optimal timing from MI to CABG surgery has been an ongoing debate, much more so in the recent years due to changes in the management of patients with ACS. There are advocates for early surgery, citing results from institutional reports with low peri-operative mortality. As previously mentioned, De Wood and colleagues advocated early surgery within 6 hours of MI because mortality in their patients was low and early surgical revascularisation was associated with significant improvement in regional and global wall motion in the LV [105]. Braxton and co-workers suggested that 48 hours was an acceptable duration to perform CABG after MI [107]. In their study, in the subgroup of patients with STEMI, surgical mortality was 50% when CABG was performed within 48 hours of STEMI compared to a mortality of 7.7% when surgery was performed more than 48 hours after STEMI. At the same time, there are also advocates for delayed surgery. Weiss and co-workers showed the temporal trend in mortality with time from MI to surgery, with maximal mortality within the first 24 hours [71]; Lee and co-workers showed that high mortality was seen in patients undergoing CABG within 3 days of MI [101]; Voisine and associates showed that in patients undergoing CABG within the first 24 hours of MI, mortality was around 10% or higher [106]. However, it is rather difficult to compare these reported series. All studies were retrospective studies. The patients in De Wood's series were considerable younger, with a mean age of 53 years, compared to the other studies, with a mean age of around 60 years. The study period varied between studies; some were carried out in the early 1990s, some in the early 2000s and some ranged from 1990s to 2000s. The pathology also differed between series; some only considered STEMI, whilst others were all-inclusive. If indeed duration between MI and CABG surgery is a predictor of higher mortality, the cause may be multi-factorial such as presence of co-morbidity in patients, underlying pathology with its associated biological response such as systemic inflammatory response and haemodynamic status. Hence, with our animal model, we attempted to remove all the other variables that might cause cardiac dysfunction in this setting and focus on purely the biological response and effect in the myocardium when subjected to regional infarction initially, before subsequent global ischaemia-reperfusion. As mentioned in Chapter 1, Section 1.3.3, Dixon and co-workers have previously shown there was up-regulation of MT1-MMP in the heart subjected to surgical ligation of coronary artery, even 3 weeks after

the infarction [42], which correlated with decreased regional stroke work. To our knowledge, our study is the first animal model study of the effects of global ischaemia-reperfusion on previously infarcted hearts, with particular attention to the influence of timing between the ischaemic injuries. The MI model in rats used here allowed a proper and simplified way of investigating purely the contribution of biological factors in causing heart dysfunction in the infarcted myocardium when it is further stressed by another ischaemic burden. The main aim of these studies was to assess the impact of 2 consecutive ischaemic burdens, as well as the interval between the insults on the capacity of the heart to recover its mechanical function. Given the evidence available so far in the literature, it will be prudent to study an early and late timing before imposing a second global ischaemia. Due to logistical constraint during the initial period of these studies, 7 days was the shortest time possible between induction of surgical MI and ex-vivo heart perfusion. This was due to the fact that surgery had to be perfomed in a different biological services unit (BSU) on a separate campus (due to refurbishment of BSU in the department), with only a weekly transport of animals between the BSUs. The 3-day infarct protocol was only carried out towards the end of the study period once the construction work was completed. For these studies, it was decided that sham operation was not necessary as our previous studies at 7-days had demonstrated that hearts from the sham group were almost identical to normal hearts.

In all groups, the development of contracture started after a similar period of global ischaemia, reaching peak contracture shortly after that. It is surprising that the infarct groups, both early and late, seemed to have a lower peak contracture compared to either normal or sham hearts. With reperfusion, there was an early and brief period of hypercontracture in all hearts, consistent with the generation of reactive oxygen species (ROS), resynthesis of adenosine triphosphate (ATP) in the presence of elevated calcium concentration and induction of mitochondrial permeability transition pore[108]. However, with sustained reperfusion, the diastolic pressure of all groups seemed to improve somewhat and be comparable again. It was once thought that contracture indices in isolated heart models implied severity of ischaemic and irreversible myocardial injury, but recent studies disputed this [109]. Preconditioning and cardioplegia affects contracture in different ways, yet recovery of myocardial contractility remained similar. Contracture reflects the changes in the metabolic state and activity in the myocytes. When ATP

levels are depleted to around 12 μmoles/g dry weight, it seemed to trigger development of contracture in the myocardium [110], with the suggestion that availability of calcium affected the peak of contracture. Different metabolic activity in normal and ischaemic myocardium may explain the differences in peak contracture developed that we observed in the normal and infarcted hearts. It would, therefore, be interesting to study the tissue metabolites in these groups to determine if there was any difference in high-energy phosphates. Our study was also only a short-term study and it would be interesting to see whether there is any long-term consequence in remodelling of the ventricles.

The recovery of LVDP we found in the study demonstrated that infarcted myocardium has less capacity for recovery of global function following another global ischaemia-reperfusion injury, but, unexpectedly only in the 7-day infarct hearts. The rate of recovery in the 7-day infarct group was slower compared to normal, 3-day infarct or sham hearts with a lower maximal recovery of the LVDP. In our study, we used LVDP as an index of global LV function. This suggests that the duration between the ischaemic insults has an impact on recovery of mechanical function of the heart. The 3-day infarct hearts may have been protected by the second window of protection of ischaemic pre-conditioning [111]. Various signalling pathways have been shown to be activated by ischaemia-reperfusion injury [75, 112]. Other than signalling pathways, there could also be activation of various bioactive molecules (such as MMP2) and molecular events that are time-dependent. It could also imply that function of bioactive molecules or its activation depends on the duration after the insult. As described in Chapter 1, optimal timing of MMP inhibition may be beneficial in remodelling of the LV, but prolonged inhibition may have the opposite of the desired effects. Besides LVDP, the recovery of CF was the other parameter that was different between the groups; however, this time, it was significantly lower in the 3-day infarct hearts compared to normal hearts. This could imply that there was more endothelial dysfunction with increased vascular tone in the early period after infarction. The balance between vasoconstriction and vasodilation in the endothelium may have changed between 3 and 7 days of infarction. Again, this could be explained by differential activation or the temporal effects of MMP2 as it can both vasoconstrict and vasodilate.

In summary, studies described in this chapter demonstrated clearly that first of all, the infarcted myocardium had less capacity to recover its contractile function when subjected to a further period of global ischaemia. The timing of the second insult also had an impact on the ability of the myocardium to recover. There must be biological factors at play in the myocardium to explain the results we found. Perhaps MMP2 has a role in causing the dysfunction seen in the 7-day infarct hearts. What is the activity of MMP2 in the infarcted myocardium during reperfusion after the subsequent global ischaemia? Could it be that MMP2 may be beneficial to the infarcted hearts during the early days of infarction? As explained earlier, we would have liked to move on to assess myocardial MMP2 activity in both the 3-day infarct hearts and 7-day infarct hearts to ascertain whether a temporal difference in myocardial MMP2 activity may be a possible explanation to the phenomenon we see, but unfortunately, due to circumstances beyond our control (with the upgrading on BSU within the department), it was not possible to do that. In subsequent chapters, we proceeded to study myocardial MMP2 activity in 7-day infarct hearts initially in an attempt to answer some of these questions.

5 MYOCARDIAL MMP2 ACTIVITY DURING AN ACUTE ISCHAEMIA-REPERFUSION PHASE

5.1 Introduction

In Chapter 4, we demonstrated that infarcted myocardium had less capacity to recover contractile function when subjected to further global ischaemia-reperfusion in comparison to normal hearts subjected to the same global ischaemia-reperfusion. The inability to recover contractile function is a manifestation of intracellular and molecular events occurring within the myocytes with ischaemia-reperfusion. With ischaemia, anaerobic metabolism will rapidly take over (resulting in intracellular acidosis and loss of contractility), with gradual depletion of ATP and ultimately causing irreversible cell and microvascular injury [8]. The mechanism of myocyte death is due to a combination of necrosis, apoptosis and autophagy [72], secondary to the role of calcium (Ca^{2+}) overload and generation of ROS both during the ischaemia and reperfusion phase of the injury. During ischaemia, intracellular acidosis results in an increase in intracellular sodium (Na^{+}), in exchange for the extrusion of excessive protons (H^{+}) generated during ischaemia [72]. This, in turn, causes a reversal of the sodium-calcium exchanger (NCX) leading to a rise in intracellular Ca^{2+}. With reperfusion, intracellular Ca^{2+} can return to normal gradually, oscillate or remained persistently high, depending on the myocyte ATP level, intracellular Na^{+} concentration and ryanodine channel release in the sarcoplasmic reticulum. Persistently raised Ca^{2+} concentration is the culprit in causing irreversible cell injury. The other major culprit of cell death is ROS. ROS is generated mainly during reperfusion with the introduction of oxygen [72]. ROS can directly or indirectly result in myocardial damage and cell death [108]. ROS interacts directly with lipids and proteins, resulting in alteration of ion channels, leading to Na^{+} and Ca^{2+} overload and ultimately cell death. Indirectly, ROS may activate signaling pathways, such as mitogen-activated protein kinases (MAPK).

As described in Chapter 1, Section 1.3.3, MMP2 has been implicated in acute ischaemia-reperfusion injury of the heart. MMP2 could be linked to ischaemia-reperfusion in various ways. Signalling pathways activated by ROS, such as MAPK, could in turn activate MMP2 and MMP9 [113]. In an animal model of repetitive ischaemia

(RI), it was shown that p38 MAPK was maximally activated on day 3 of the RI protocol [114] and MMP2 and MMP9 were also activated by the RI protocol, specifically on day 3 as well [113]. When the animals were treated with p38 MAPK inhibitor for 2 days during the RI protocol, there was significant reduction in the expression and activation of MMP2 and MMP9 [113]. Other than signalling pathways, ROS may also directly activate MMPs. Peroxynitrite, $ONOO^-$, one of the species of ROS, has been shown to directly activate MMP2 in myocytes, resulting in myocardial injury [79].

Once activated, MMP2 could affect processes and structures within the myocytes, thus contributing to myocardial injury. For example, in cancer cell culture studies, downregulation of MMP2 was consistently shown to induce apoptosis [115, 116]. Extensive research into acute ischaemia-reperfusion injury has shown that mitochondria are very important regulators of cell energetics and viability [72]. Experimental evidence suggests that MMP2 affects the architecture and function of mitochondria. Transgenic mice over-expressing MMP2 were shown to have disruption to their mitochondrial structure [117] and MMP2 inhibition studies in bovine retinal endothelial cells showed that MMP2 inhibition was associated with decreased mitochondria superoxide production and membrane permeability [118]. When transgenic mice overexpressing MMP2 were subjected to acute ischaemia-reperfusion, the recovery of myocardial function was worse compared to wild-type, with evidence of disturbed mitochondrial respiration and mitochondrial deformation associated with mitochondrial swelling and loss of integrity of membrane and cristae [119]. Zhou and co-workers also demonstrated that in a baseline, unstressed state, there was no difference in the haemodynamic parameters between the transgenic mice over-expressing MMP2 and wild type. However, when subjected to acute ischaemia-reperfusion, transgenic mice were more vulnerable and susceptible to injury, with less recovery capacity. These studies suggest that MMP2 may have an acute role in myocardial ischaemia-reperfusion injury.

We, therefore, wanted to investigate the role of MMP2 in the increased vulnerability seen in the 7-day infarct hearts carried out in studies described in Chapter 4. In order to establish any link between MMP2 activity and the cardiac dysfunction observed, we determined the temporal effect of ischaemia-reperfusion on MMP2 activity during isolated heart perfusion, using different MMP2 activity assays.

5.2 Methods

5.2.1 MMP2 activity (assessed using MMP2 activity assay) in myocardium (tissues) during acute ischaemia-reperfusion

MMP2 activity was measured in isolated hearts from normal, infarcted or sham rats. Rats were subjected to LAD ligation as described in Chapter 2, Section 2.1 and the sham hearts were subjected to the same surgery (up to and including the stage where the heart was exteriorised) as the LAD ligation hearts except that suture ligation of LAD was not carried out. All the animals that underwent surgery were recovered for 7 days, with free access to food and water. After 7 days, 3 groups of hearts (normal, infarcted and sham) were perfused using the isolated Langendorff heart perfusion as described in Chapter 2, Section 2.2, with the perfusion protocol as described in Chapter 4, Section 4.2.1, depicted in Figure 4.1. The hearts were freeze-clamped with liquid nitrogen at different time points during the perfusion protocol to collect myocardial tissues for analysis (n=3 in each group at each time point). The chosen times were: (i) after 20 min of aerobic equilibration (baseline), (ii) at the end of 30 min of normothermic global ischaemia (0 min), (iii) 2 min of aerobic reperfusion after 30 min of normothermic global ischaemia (2 min), (iv) 5 min of aerobic reperfusion after 30 min of normothermic global ischaemia (5 min), (v) after 10 min of aerobic reperfusion after 30 min of normothermic global ischaemia (10 min).

The tissues collected were stored at -80°C until use. The hearts were homogenised as described in Chapter 2, Section 2.3. The supernatant was collected and used to assess MMP2 activity using the MMP2 activity assay (GE Healthcare, Amersham, UK). The assay was carried out as described in Chapter 2, Section 2.7. The results of the assay were expressed as ng/ml/gm of protein. Statistical analysis was carried out with ANOVA test between the groups at different time points, using post-hoc Tukey's test for multiple comparison. A value of $p<0.05$ was considered statistically significant.

5.2.2 MMP2 release (assessed using substrate zymography) in the coronary effluent at reperfusion after global ischaemia

Normal hearts and infarcted hearts were used in this study. Infarcted hearts were harvested from rats subjected to LAD ligation as described in Chapter 2, Section 2.1, and allowed to recover for 7 days prior to isolated heart perfusion. The 2 groups of hearts were perfused in the isolated Langendorff heart perfusion as described in Chapter 2, Section 2.2, with the perfusion protocol as described in Chapter 4, Section 4.2.1, depicted in Figure 4.1. Coronary effluent was collected (6 mls) in each group after 20 minutes of aerobic equilibration and immediately at reperfusion following 30 minutes of normothermic global ischaemia (n=6 in each groups). Cheung and co-workers had demonstrated that the release of MMP2 peaked within the first 2 min of reperfusion after global ischaemia [35], and the volume of effluent collected for their analysis was 6 mls. Hence, I decided to collect 6 mls of effluent for analysis. The time taken to collect 6 mls of effluent at aerobic perfusion averaged at 40 s and immediately after ischaemia was 90 s.

The effluent was concentrated using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (MerckMillipore, UK), centrifuged at 4000g in 4°C for 10 minutes. The final volume of the concentrate was between 200-250μl (measured using graduation in the filter unit). The concentrate was analysed for protein content using the Bradford protein assay as described in Chapter 2, Section 2.4 and used to assess gelatinolytic activity using substrate zymography. The presence of MMP2 in the effluent was also confirmed using protein (Western) immunoblot as zymography is not a specific technique for MMP2. Western blotting was carried out as described in Chapter 2, Section 2.5. Quantitative analysis was not performed with Western blotting, since the aim was to confirm the presence of MMP2 by detection of the relevant molecular bands at both 72 and 62 kDA.

The sample collection and analysis with zymography was carried out on the same day to protect the integrity of the proteins, by preventing freeze-thaw cycle. Coronary effluent concentrate, 30 μl, was used in gelatin zymography. Zymography was carried out as described in Chapter 2, Section 2.6, with the reagents and incubation times as detailed. Gelatinolytic activities were detected as clear bands corresponding to molecular weights

of the enzymes. The destained gels were scanned using GenSnap software and band density measured using Image J software. Statistical analysis between 2 groups was carried out using Student's t-test. A value of $p<0.05$ was considered significant statistically.

5.3 Results

5.3.1 MMP2 activity (assessed using MMP2 activity assay) in myocardium (tissues) during acute ischaemia-reperfusion

The activity of MMP2 in infarcted heart tissues was higher at all the time points assessed compared to normal or sham hearts (Fig 5.1). After aerobic perfusion (baseline stability period), MMP2 activity in infarcted heart tissues was significantly ($p=0.04$) higher (at 0.87±0.08 ng/ml/gm of tissue) compared to 0.63±0.02 and 0.61±0.06 ng/ml/gm in normal and sham groups, respectively. MMP2 activity peaked at 5 minutes of reperfusion in the infarcted myocardium (1.03 ng/ml/gm of protein), which was significantly ($p=0.02$) higher than that in the normal myocardium (0.576 ng/ml/gm of protein).

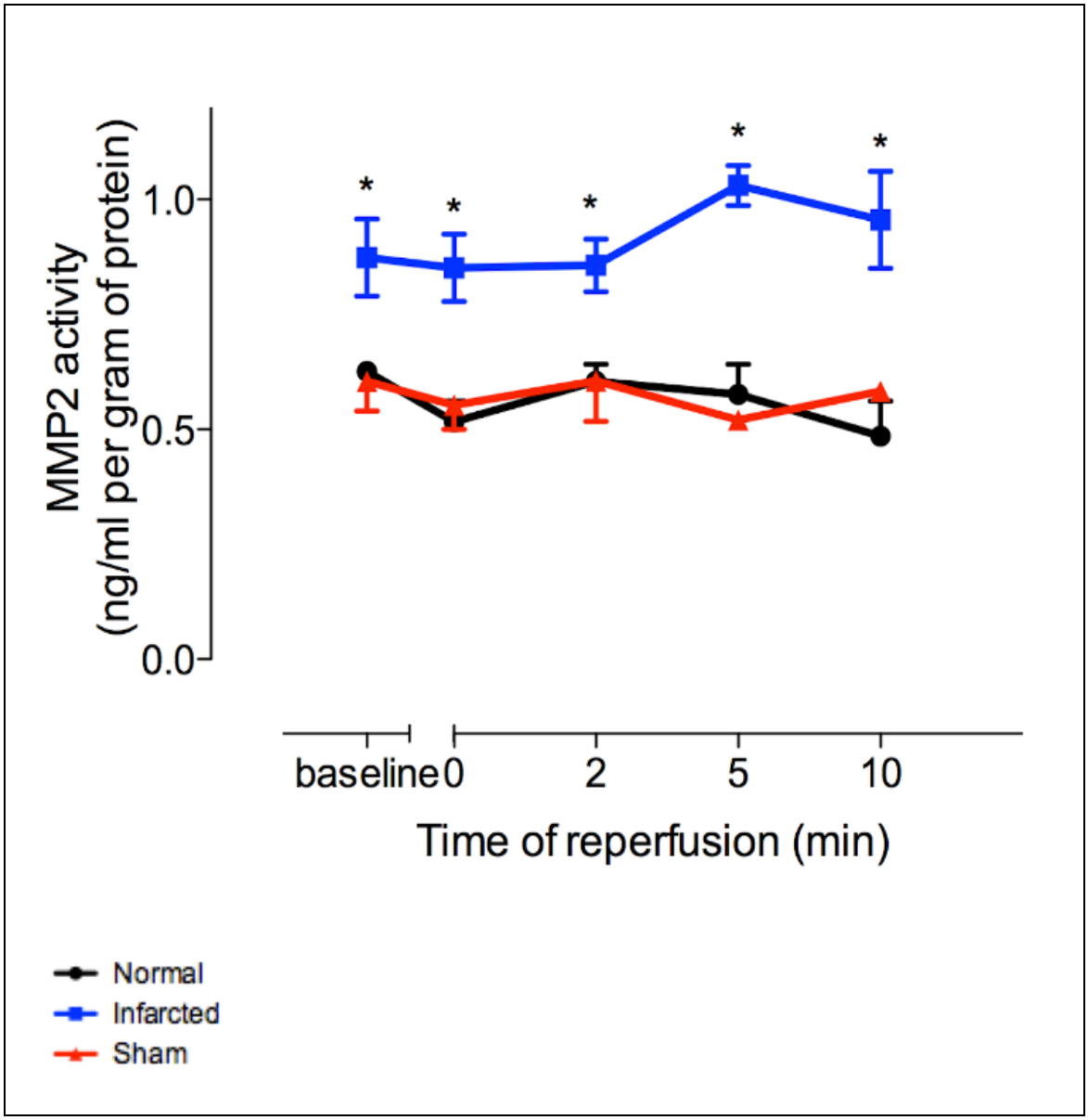


Figure 5.1 MMP2 activity in normal, infarcted and sham hearts. n=3 in each group at each time point.

MMP2 activity in normal, infarcted and sham hearts at the end of baseline, aerobic perfusion and immediately after global ischaemia (0), and at 2, 5, and 10 min of reperfusion after 30 min of global ischaemia. * p<0.05 vs normal or sham hearts.

5.3.2 MMP2 release (assessed using substrate zymography) in the coronary effluent at reperfusion after global ischaemia

The presence of MMP2 in the effluent of normal and infarcted hearts at the end of aerobic perfusion and immediately at reperfusion was confirmed by the detection of bands at 72 and 62 kDA (the molecular weights for pro-MMP2 and MMP2, respectively) using protein immunoblot. In the coronary effluent, MMP2 activity was detected at 72 and 62 kDa, by comparison with MMP2 standard (Calbiochem, Nottingham, UK). In normal hearts, 30 min of global ischaemia increased MMP2 activity in the initial 6 mls of reperfusion effluent to a significantly higher value (0.63±0.06 arbitrary unit (AU) vs 0.28±0.08 AU, p=0.02) than that of baseline perfusion. MMP2 activity in the coronary effluent of infarcted hearts was higher at both 72 (p=0.003) and 62 (p=0.0005) kDA compared to the coronary effluent of normal hearts at baseline aerobic perfusion (Fig 5.2). However, after 30 min of global ischaemia, there was no further increase in MMP2 activity in the effluent of infarcted hearts. The release of MMP2 into the initial 6 mls of effluent of both normal and infarcted hearts was at comparable levels after 30 min of global ischaemia.

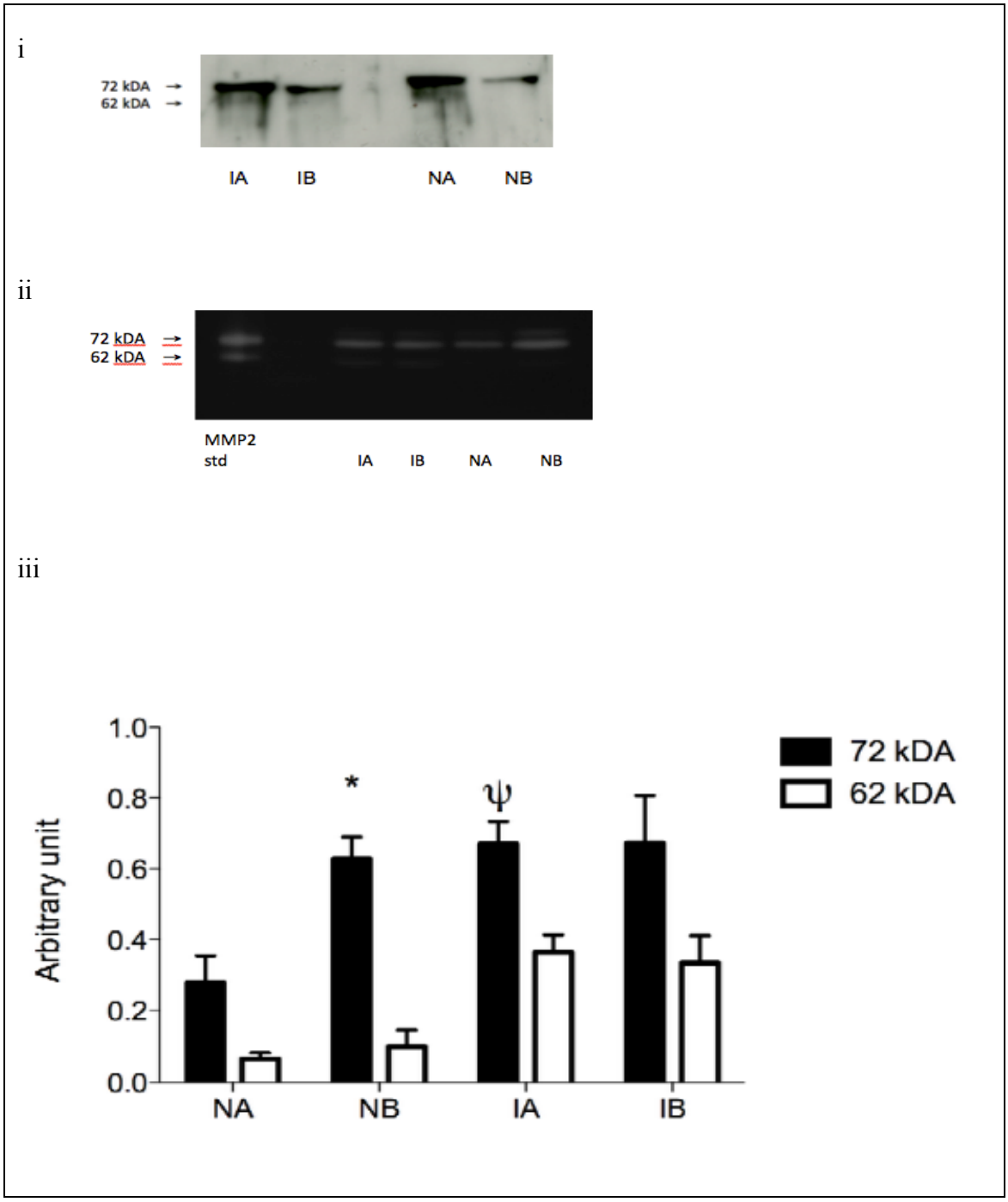


Figure 5.2 MMP2 activity in coronary effluent of normal and infarcted hearts during aerobic perfusion and after 30 min of global ischaemia. n=6 in each group.

(i) Representative Western blots confirming the presence of MMP2 in the coronary effluent of infarcted and normal hearts at baseline aerobic perfusion and after 30 min of global ischaemia. (ii) Representative zymogram of coronary effluent compared to commercially available MMP2 standard. Gelatinolytic bands detected at 72 kDA in all samples, with fainter bands at 62 kDA (iii) Densitometric analysis of gelatinolytic activities in coronary effluent. NA – effluent in normal hearts at baseline aerobic perfusion; NB – effluent in normal hearts after global ischaemia; IA – effluent in infarcted hearts at baseline aerobic perfusion; IB – effluent in infarcted hearts after global ischaemia. * $p<0.05$ vs NA, ψ $p<0.05$ vs NA

5.4 Discussion

It would be ideal to be able to analyse heart tissue homogenates for MMP2 activity in its unaltered endogenous state, and its molecular interactions intact. However, this was difficult to achieve as the extraction processes, together with various reagents used in the process, will interact and may alter the physiological state of MMP2. Although DTT should be excluded from the extraction buffers due to its property as a reducing agent, the results obtained in Chapter 2, Section 2.3 clearly showed that without DTT, the yield of protein extraction was so poor that it may not be possible to perform any assay of MMP2 activity at all. The final concentration of DTT added to the buffers was only a fifth of the usual concentration used to extract proteins from tissues for immunoblotting purposes. This was thought to be a reasonable compromise. Samples from different groups were processed under identical conditions and, although this may not truly reflect physiological state, it will still allow valid comparisons of MMP2 activity in different groups of hearts to establish a proof of principle.

The most widely used and conventional method to assess MMP2 activity is substrate zymography. This is an electrophoretic technique based on SDS-PAGE, where the substrate is co-polymerised with the polyacrylamide gel; hence, detection of MMP activity is based on molecular weight separation. It is relatively cheap, sensitive, widely used (hence allowing comparison with other studies) and is a good screening tool. As gelatinolytic activities are detected based on molecular weight (and not from specific MMP2 antibody), it may not be entirely accurate, especially in the light of recent mounting evidence that MMPs can also be activated by mechanisms other than proteolysis, such as s-nitrosylation and phosphorylation [20, 27, 28]. Because of these limitations in zymography, a new colorimetric assay was also used in this study to measure MMP activity. This is a recently developed method, and uses modified pro-urokinase; it acts as a pro-form of the detection enzyme, which is activated by active MMP2 [120 121]. It is described by the manufacturers (GE Healthcare) to be a precise, accurate and quantitative technique, that is also specific for MMP2. However, it is considerably more expensive than substrate zymography and has a limited shelf-life once the kit is opened; for this reason, studies described in Chapter 2, Section 2.7 were performed in an attempt to validate this assay. Despite strict adherence to

manufacturer's instructions, the data show that there was considerable variance in the standard curve. Therefore, caution should be exercised in interpreting the absolute quantitative values obtained with the assay, especially when the assays were carried out on different plates and/or on different days. However, a comparison of trend between different groups (if all the samples were analysed on the same plate, with the assay carried out at the same time) could still be examined. Despite these disadvantages, the assay was still used as it seems to be a more accurate assay of MMP2 activity compared to substrate zymography, and it is readily performed in the laboratory. It measures active MMP2 based on its activity (and not just presumed active based on it molecular weights). The assay consists of 96-well plate, which allows simultaneous comparison of the different myocardial samples and hence allowing us to assess if there were any differences in the trend of activity between the different groups.

It is important to establish the temporal relationship between MMP2 and the pathophysiology in question. In tumorigenesis, the link between MMPs and the type and stage of cancer is important. In a murine model of kidney glomerular disease, MMP2 and MMP9 were shown to be detrimental in the early stage of the disease but protective in the advanced stage [122]. In cardiovascular diseases, high MMP3 activity prevented plaque formation but once the plaque is formed, high activity contributes to plaque instability, leading to rupture [123]. In studies of heart failure using LAD ligation models in rats, it was found that the protein level and expression of MMP2 were both significantly higher than pre-infarction as early as day 1 post-ligation [33], and remained elevated for about 2-3 weeks post MI. After this, both mRNA and protein level gradually decreased towards baseline. However, there was a second surge in protein level of MMP2 at 10 weeks. Our results concurred with this study, showing that MMP2 activity in the myocardium was also higher in previously infarcted hearts 7 days after LAD ligation compared to normal or sham-operated animals when measured during baseline aerobic isolated Langendorff perfusion. Another ischaemic insult was superimposed on the infarcted hearts during the period where both activity and expression of MMP2 was high, but there was no further surge in MMP2 activity as demonstrated by a relatively constant level of activity in the infarcted hearts during the acute reperfusion phase. This could suggest that there was maximal activation of MMP2 after the initial infarction, which was sufficient to reduce the capacity of the infarcted

myocardium to tolerate further ischaemic insult. Release of MMP2 into the coronary effluent supported this conclusion. For normal hearts, there was an increased MMP2 release into the effluent after 30 min of global ischaemia, concurring with the results reported by Cheung and associates [35]. In their study of acute ischaemia-reperfusion in isolated Langendorff rat heart perfusion, they found a significant increase in MMP2 in the coronary effluent in a time-dependent manner during reperfusion after 20 min of global no-flow ischaemia, with a peak of MMP2 in the effluent collected at 1 min of reperfusion. Interestingly, the release of MMP2 into the coronary effluent of infarcted hearts was the same during baseline aerobic perfusion and after 30 min of global ischaemia; the significance of this is not entirely clear. One alternative explanation may be that the number of hearts used was too small to detect any change in activity level. The other possible reason is that there may already be maximal activation of MMP2 with the initial infarction, so that subsequent ischaemia did not have any further effects on MMP2 activation.

In conclusion, the data presented in this chapter showed that in infarcted hearts, baseline MMP2 activity during aerobic perfusion was significantly higher compared to either normal or sham hearts. When subjected to another global ischaemic insult, although there was no further increase in myocardial MMP2 activity during reperfusion, there was significant impairment in the recovery of mechanical function of the hearts. Increased MMP2 activity during baseline perfusion could be the culprit of heart dysfunction seen here. If MMP2 is indeed a contributory factor, inhibition of MMP2 should ameliorate the heart dysfunction. This formed the basis of investigation in the subsequent chapter.

6 THE EFFECTS OF PHARMACOLOGICAL MMP2 INHIBITION

6.1 Introduction

The data presented in the preceding chapter established the temporal profile of myocardial MMP2 activity in infarcted hearts during a subsequent acute ischaemia-reperfusion period. During this insult, there was detrimental effect to the recovery of the mechanical function of the infarcted hearts compared to normal or sham, making the previous infarction an independent variable in causing a degree of dysfunction. MMP2 activity was simultaneously shown to be elevated in infarcted hearts compared to normal or sham hearts during the subsequent acute ischaemia-reperfusion period, raising the possibility of MMP2 as a contributory factor to the mechanical dysfunction seen. To determine the possibility of a causal relationship, pharmacological inhibition of MMP2 activity may ameliorate myocardial dysfunction.

MMP inhibition could be targeted with different strategies [124]. The endogenous inhibitor, TIMP, is a potential candidate but it is difficult to control the balance between MMP/TIMP activities. Besides, TIMP binds with different rate and affinity to different MMPs and the control of these are difficult to achieve. TIMP may also activate some MMPs, rather than inhibiting it; TIMPs are more than just endogenous MMP inhibitors, they are bioactive molecules that can affect processes such as cell growth and death independently from its inhibitory actions on MMPs [12]. In terms of exogenous inhibitors, first generation synthetic inhibitors of MMPs, such as batimastat, are small molecules, which are potent broad inhibitors; the mechanism of inhibition is via its zinc-chelating ability. Improved characterisation of the structure of MMPs with crystallography allowed more refined second and third generation inhibitors to be synthesised. Even so, clinical trials using these inhibitors for example prinomastat, mainly in cancer trials, were disappointing [122, 123, 125]. The main problem with MMP inhibitors in clinical trials was disabling musculoskeletal side effects experienced by many patients, resulting in early termination of these trials. Besides, the inhibitors tested were broad inhibitors and it was thought that perhaps protective effects of MMPs such as repression of tumour angiogenesis may have also been indiscriminately inhibited.

Besides the synthetic inhibitors, the tetracycline family of antibiotics, have been shown to inhibit MMPs. Of this family of antibiotics, doxycycline is a good example and has been used extensively. Doxycycline is a commonly used antibiotic to treat various bacterial infections, with a long history of clinical use and good safety margins. Doxycycline is the most potent MMP inhibitor of the tetracycline family of antibiotics [126]. The mechanism of MMP inhibition is independent of its antibacterial action, namely via the metal ion (zinc and calcium) β-diketone moiety [127]. It inhibits enzyme activity by chelating the active Zn^{2+} ion. Currently, a sub-antimicrobial dose of doxycycline is the only MMP-inhibitor approved by the Food and Drug Agency (FDA) for clinical use. In cardiovascular diseases, there are numerous studies of long-term LV remodelling using doxycycline as a MMP inhibitor [46-49, 51]. Doxycycline was also shown to be an effective MMP2 inhibitor in experimental studies of acute ischaemia-reperfusion injury [35, 38]. Doxycycline makes a compelling choice for our experimental studies, due to the ease of translating its use as MMP2 inhibitor from bench to bedside, if our data supported its cardioprotective effect in this setting of acute ischaemia-reperfusion. There would be no need to conduct Phase I trials to determine safety of the drug.

In order to establish any causal relationship between MMP2 and mechanical dysfunction seen in the infarcted hearts following global ischaemia, we also consider other alternative inhibitors. The primary consideration in choosing the second inhibitor was that it has to be an established inhibitor, with effective inhibition demonstrated experimentally. The primary goal is to enable us to demonstrate proof-of-principle regarding the role of MMP2 in this experimental setting of acute ischaemia-reperfusion. Hence, we decided on using 1,10-phenanthroline. It is again a commonly used MMP inhibitor in many studies [35, 38, 128], with proven effectiveness in inhibiting MMP2. 1,10-phenanthroline is a heterocyclic organic compound that inhibits MMPs by chelating the Zn^{2+} ion and rendering the enzyme inactive. The chelating and enzyme inhibitory effects were first observed in carboxypeptidase A [129]. The inhibition is fully reversible on dilution or addition of Zn^{2+} ions. It has a much lower affinity for calcium.

The final part of these studies was to demonstrate that any improved mechanical function in isolated hearts, with addition of MMP inhibitor in the perfusate, was associated with biochemical evidence of reduced myocardial MMP2 activity. It is not possible to carry out a tissue assay of MMP2 activity when 1,10-phenanthroline is

present as it is a reversible inhibitor and the process of tissue extraction will dissociate the complexes formed and reverse the inhibitory action of 1,10-phenanthroline. Therefore, it was crucial to find an irreversible inhibitor to perform this aspect of the study, and one such agent [a commercially available MMP2/MMP9 inhibitor V, 444285 (4-(4-(Methanesulfonamido)phenoxy)phenylsulfonyl)methylthiirane] was sourced from Calbiochem (Nottingham, UK); it is a cell-permeable compound, with enhanced aqueous solubility and improved selectivity for MMP2 (K_i =16 nM for MMP2, much higher for other MMPs). In a comparison study of different synthetic MMP inhibitors, 444285 was found to be a potent irreversible MMP2 inhibitor [130]. We conducted an initial dose-response study with 444285 to determine an optimal concentration, and subsequently, carried out a myocardial activity assay to determine if any improved recovery was due to biochemical myocardial inhibition of MMP2 activity.

6.2 Methods

6.2.1 Effect of doxycycline in normal hearts subjected to acute ischaemia-reperfusion injury

Adult, male Wistar rats (250-350g) (n=5-6/study) were perfused using the isolated Langendorff heart perfusion as described in Chapter 2, Section 2.2. The hearts were equilibrated for 20 min with aerobic perfusion, followed by 30 min of global normothermic ischaemia (induced by clamping of the aortic in-flow line). Subsequently, the hearts were reperfused for a further 60 min with aerobic perfusion. Two treatment groups were examined with different protocols of 100 μM doxycycline treatment (Fig 6.1), and they were compared to a historical control group of normal hearts (defined as Group i in Fig 6.1) carried out in studies described in Chapter 4 with the same perfusion durations. The doxycycline concentration of 100 μM was used as it had been demonstrated in other studies to be effective in inhibiting MMP2, using a similar model and perfusion protocol [35, 38]. In Group ii, doxycycline was used as a pre-treatment prior to global ischaemia, by adding it to the perfusate during the last 10 min of aerobic perfusion. In Group iii, doxycycline was added as both a pre- and post-treatment, i.e for

10 min before and after ischaemia. This was a common protocol used in other experimental isolated heart perfusion models of acute ischaemia-reperfusion studies.

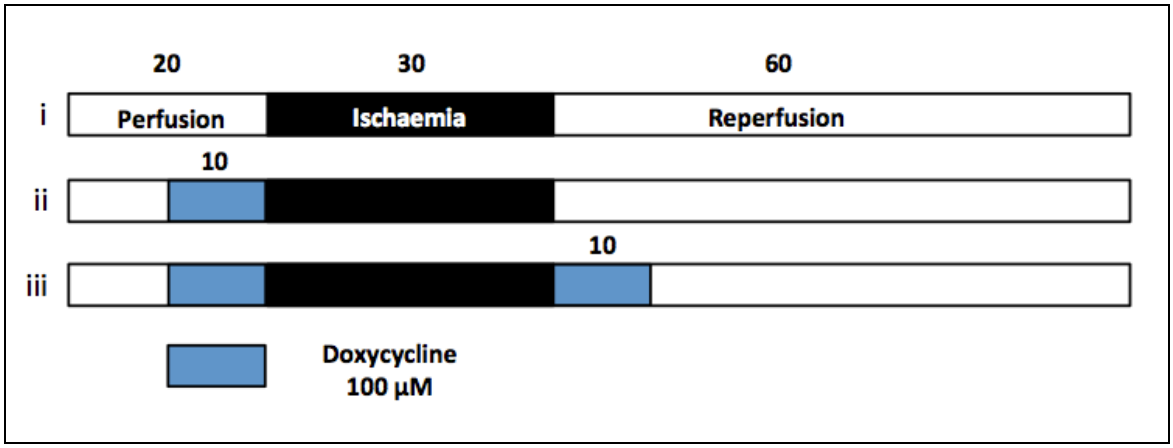


Figure 6.1 Langendorff perfusion protocol with addition of doxycycline.

Group i consisted of historical control normal hearts, perfused in studies carried out in Chapter 4 and compared with Group ii and Group iii, perfused in this Section, with the addition of doxycycline to the perfusate as shown.

The HR, LVDP and LVEDP was monitored and acquired continuously throughout the perfusion protocol. The recovery of LVDP was measured as a % recovery of its pre-ischaemic value. The recovery profile during the reperfusion period was compared to isolated heart perfusion of normal hearts that was carried out in Chapter 4. Historical control group was used to minimise the number of animals used in the experiments and to be compliant to the 3R principles set out in the Handbook of Laboratory Animal Management and Welfare [131]. The experiments for the historical control group were only carried out once I was completely competent with the technique, and the interval between the historical control experiments and these experiments was within 9 months. The recovery profile of each group was analysed using 2-way repeated measures ANOVA. The mean final LVDP recovery between the groups was also compared using ANOVA and post-hoc analysis by means of Tukey's test (for multiple comparisons). A value of $p<0.05$ was considered statistically significant.

6.2.2 Effect of 1,10-phenanthroline in hearts subjected to acute ischaemia-reperfusion injury

Adult, male Wistar rats (250-350 g) were used in these experiments. Four groups of hearts (n=5-6/group) were perfused by isolated Langendorff heart perfusion as described in Chapter 2, Section 2.2. As above, Group i consisted of normal hearts and Group iii was infarcted hearts perfused by isolated Langendorff heart perfusion with 20 min of aerobic equilibration, followed by 30 min of global ischaemia and 60 min of reperfusion as described Chapter 4. Group ii was hearts harvested from normal male rats (normal hearts) and Group iv was hearts harvested from male rats where the LAD was ligated 7 days prior to Langendorff perfusion. The anaesthesia and surgical procedure performed was as described in Chapter 2 Section 2.1. During isolated Langendorff perfusion, the hearts were first equilibrated with aerobic perfusion for 20 min, followed by 30 min of global normothermic ischaemia (induced by clamping of the aortic inflow line). Subsequently the hearts were reperfused aerobically for 60 min (Fig 6.2). 1,10-phenanthroline (100 μM) was added to the perfusate 10 min before and after ischaemia. This protocol and drug concentration was shown in previous studies to be effective in inhibiting MMP2 [35]. The HR, LVDP and LVEDP was monitored and acquired continuously throughout the perfusion protocol. CF was measured every 10 min during the perfusion period. The recovery of LVDP was expressed as a % recovery of its pre-ischaemic value. The recovery profile during the reperfusion period was compared to isolated heart perfusion of equivalent normal and infarcted hearts from studies described in Chapter 4, again as an effort to reduce the number of animals used in the study. The recovery profile was analysed using the exponential association curve. The mean final LVDP recovery between the groups with and without 1,10-phenanthroline was also compared using unpaired Student's t-test (Group i vs Group ii and Group iii vs Group iv). A value of $p<0.05$ was considered statistically significant.

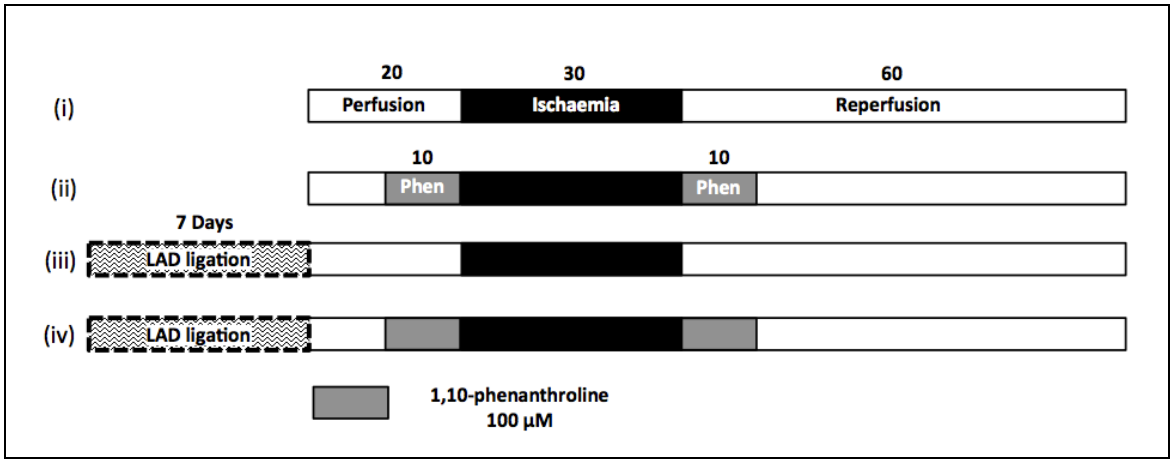


Figure 6.2 Langendorff perfusion protocol in normal and infarcted hearts, with addition of 1,10-phenanthroline.

Group i and Group iii were historical control groups, consisting of normal and infarcted hearts respectively that were perfused in studies described in Chapter 4. These groups were compared to Group ii and Group iv respectively.

6.2.3 Effect of 444285, a specific MMP2/MMP9 inhibitor, in infarcted hearts subjected to acute ischaemia-reperfusion injury

Adult, male Wistar rats (250-350 g) were used (n=4-6/group). The rats were subjected to LAD ligation as described in Chapter 2, Section 2.1 and subsequently perfused in isolated Langendorff mode as described in Chapter 2, Section 2.2. During isolated Langendorff perfusion, the hearts were first equilibrated with aerobic perfusion for 20 min, followed by 30 min of global normothermic ischaemia (by clamping of the aortic inflow cannula) and subsequently 60 min of reperfusion (Fig 6.3). The HR, LVDP and LVEDP were monitored and acquired continuously.

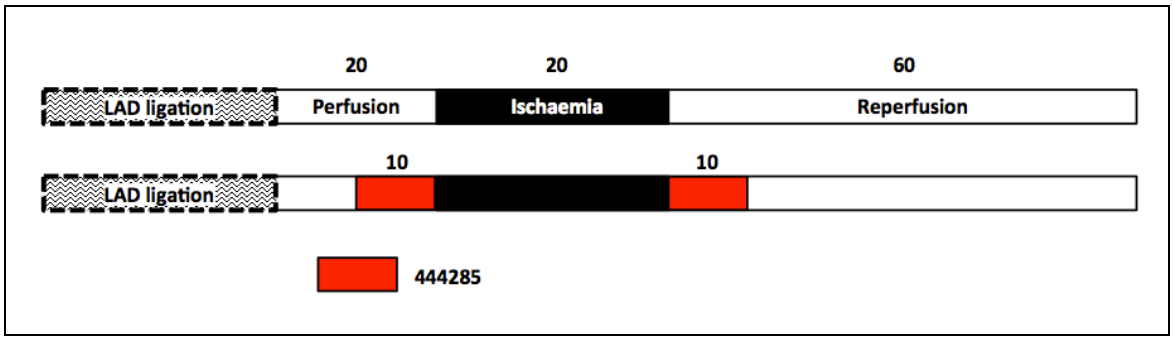


Figure 6.3 Langendorff perfusion protocol in infarcted hearts, with addition of 444285 at 50, 100, and 150 nM 10 min pre- and post-ischaemia.

To our knowledge, there are currently no published studies (either in isolated hearts or in vivo models) using 444285 as MMP2 inhibitor; therefore, we conducted a dose-response study of possible concentrations of 444285 based on the enzyme dissociation constant (K_i) of 444285, which is 16 nM. The starting concentration should be at least three-fold higher than the K_i of 444285; hence 50, 100 and 150 nM were used. The inhibitor was added to the perfusate 10 min before and after global ischaemia (Fig 6.3). The CF was measured every 10 minutes during reperfusion. The recovery of LVDP was expressed as % pre-ischaemic value. The recovery profile during the reperfusion period was compared to isolated heart perfusion of infarcted hearts as carried out in Chapter 4, Section 4.2. The recovery profile was analysed using the exponential association curve. The mean final LVDP recovery between the groups was also compared using one-way ANOVA and post-hoc test by means of Dunnett's test (for multiple comparisons). A value of $p<0.05$ was considered statistically significant.

6.2.4 Effect of 444285 on myocardial MMP2 activity in infarcted hearts subjected to acute ischaemia-reperfusion.

Adult, male (250–350 g) Wistar rats were used in this experiment. The rats were subjected to LAD ligation as described in Chapter 2, Section 2.1. All the animals were recovered for 7 days, with free access to food and water. After 7 days, the infarcted hearts were harvested from the animal and perfused in the isolated Langendorff heart perfusion as described in Chapter 2, Section 2.2. Two groups of hearts were compared in this study. The perfusion protocol was as described in Section 6.2.3 and depicted in Fig 6.3. Group 1 hearts were perfused without 444285 whilst Group 2 hearts were perfused with the optimal dose of 444285 that produced maximal recovery during reperfusion based on the dose response study carried out in Section 6.2.3. This optimal dose was added to the perfusate 10 minutes before and after global ischaemia (based on the data obtained in Section 6.3.3). The aim of using 444285 was to attempt to demonstrate biochemical evidence of myocardial MMP2 activity inhibition, which contributes to improved mechanical recovery of isolated hearts after ischaemia.

The hearts were freeze-clamped with liquid nitrogen at different time points [after 20 min of aerobic perfusion, after ischaemia (0'), 2 min of reperfusion (2'), 5 min of

reperfusion (5') and 10 min of reperfusion (10')] during reperfusion (as in Section 5.2.1) (n=4 in each group at each time point). The hearts were stored at -80°C until use. The hearts were homogenised with lysis buffer as described in Chapter 2, Section 2.3. The supernatant was collected and used to assess MMP2 activity using MMP2 activity assay (GE Healthcare, Amersham, UK). The activity assay was carried out as described in Chapter 2, Section 2.7. Protein assay was also carried out to determine the protein content in the supernatant as described in Chapter 2, Section 2.4. The result of MMP2 activity assay was expressed as ng/ml/g of protein. Mean MMP2 activity at different times in each group was compared using ANOVA, with post-hoc Tukey's test for multiple comparisons. Mean MMP2 activity at each time point between infarcted hearts without and with 444285 treatment were compared using Student's t-test. A value of $p<0.05$ was considered significant.

6.3 Results

6.3.1 Effect of doxycycline in normal hearts subjected to acute ischaemia-reperfusion injury

The baseline parameters of HR, LVDP and LVEDP for all 3 groups are shown in Table 6.1.

Table 6.1 Baseline parameters (HR, LVDP and LVEDP) obtained after aerobic perfusion in Group i (historical control group), Group ii (pre-treatment with doxycycline) and Group iii (pre- and post-treatment with doxycycline). In groups where doxycycline was added to the perfusate, the parameters were obtained prior to the addition of doxycycline.

	Group i (n=5)	Group ii (n=6)	Group iii (n=5)	
HR (beats/min)	300±24	286±8	274±6	p=0.45
LVDP (mmHg)	119±5	132±5	134±5	p=0.14
LVEDP (mmHg)	3±2	3±1	6±1	p=0.12

The addition of doxycycline to the perfusate affected the HR and LVDP, but not the LVEDP (Fig 6.4). The mean HR decreased significantly from 278±5 beats/min to

227±9 beats/min (Fig 6.4i) (p=0.0001) and the mean LVDP decreased from 133±4 mmHg to 126±4 mmHg (Fig 6.4ii) (p=0.02) after the addition of doxycycline to the perfusate during aerobic perfusion.

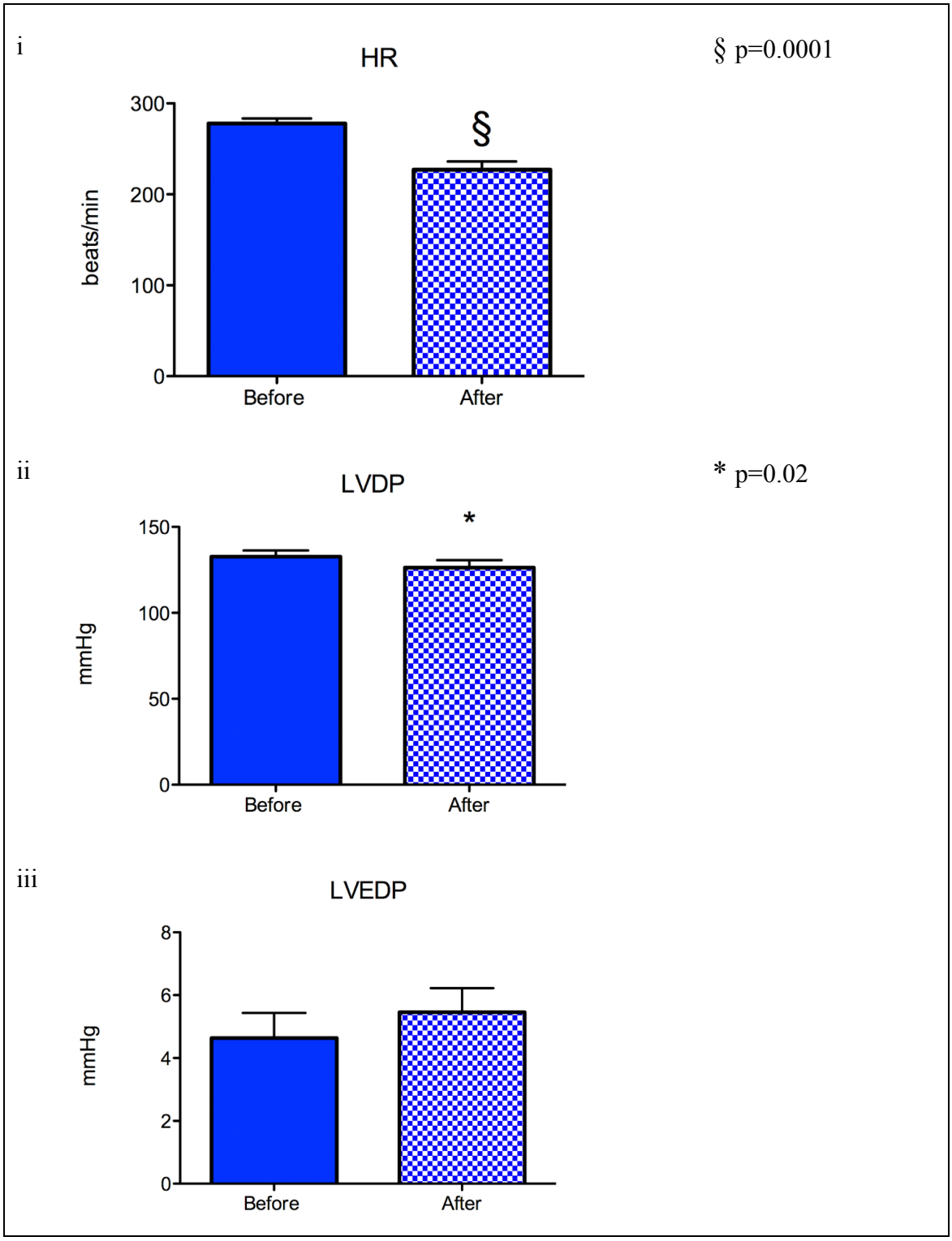


Figure 6.4 Graphs showing the change in (i) HR, (ii) LVDP and (iii) LVEDP before and 10 min after addition of doxycycline in the perfusate during aerobic perfusion of normal hearts. n=11. § p=0.0001, * p=0.02

There was slow and gradual recovery of the LVDP in all groups throughout the reperfusion period (Fig 6.5). However, recovery in hearts perfused with doxycycline (Groups ii and iii) was slower compared to the normal hearts (Group i) perfused in studies described in Chapter 4, Section 4.3.2.

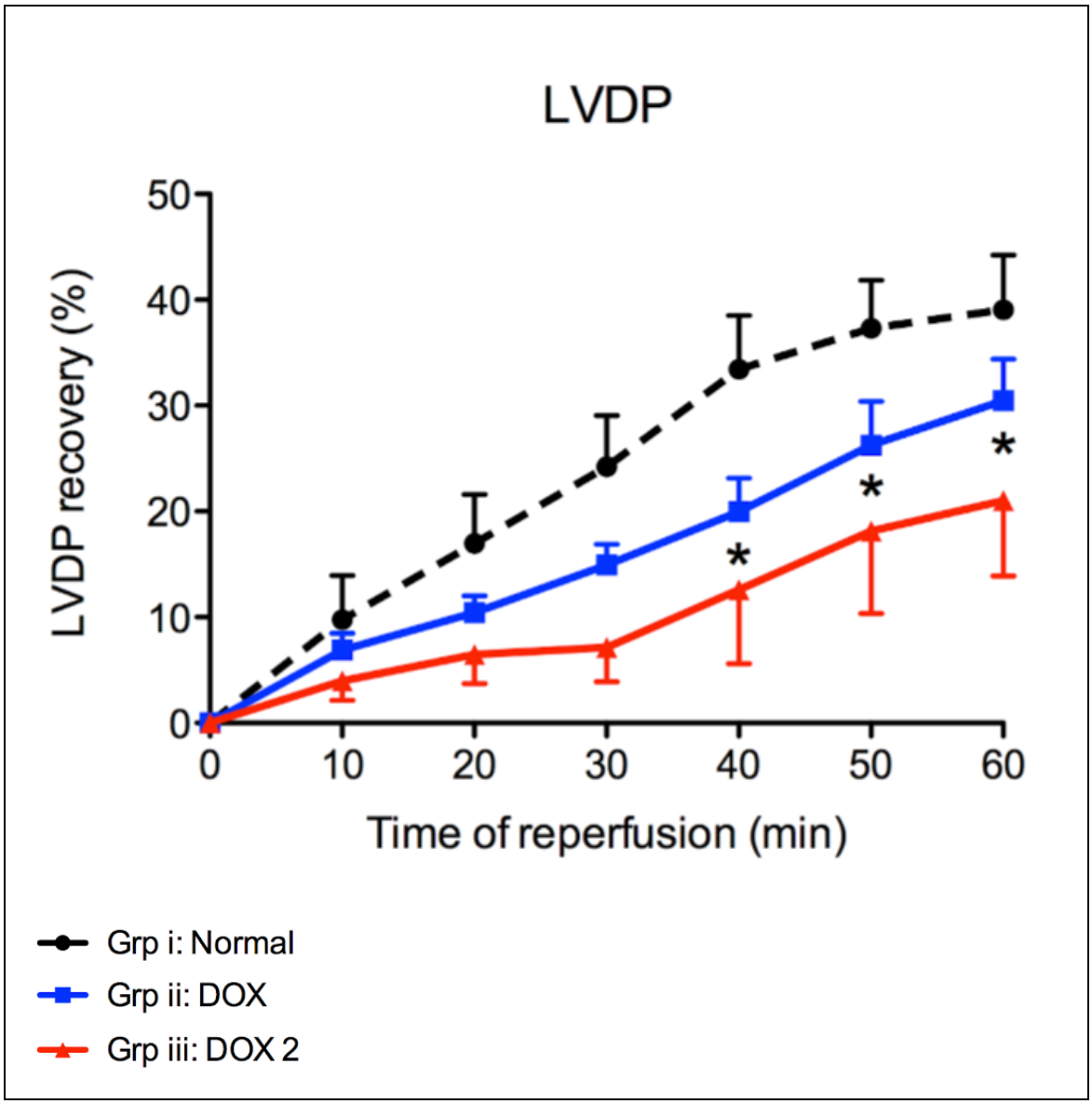


Figure 6.5 Recovery profile of LVDP in normal hearts during reperfusion after 30 min global ischaemia, without and with addition of doxycycline to the perfusate.

Group i: Control normal hearts, n=5; Group ii: Normal hearts perfused with 100 µM doxycycline during the last 10 min of aerobic perfusion, n=6; Group iii: Normal hearts perfused with 100 µM doxycycline for 10 min before and 10 min after ischaemia, n=6. * p<0.05

The recovery of LVDP in Group (i) reached a plateau towards the end of the reperfusion period, but the recovery of LVDP in the hearts in Groups (ii) and (iii) seemed to be increasing still, even at the end of perfusion. From 40 min of reperfusion onwards, the recovery of LVDP in Group (iii) was significantly lower compared to Group (i) ($p<0.05$). The final recovery achieved (% pre-ischaemic LVDP) was 39±5%, 30±4% and 21±7% ($p=0.1$) in Groups (i), (ii) and (iii) respectively.

The recovery of HR in hearts perfused in Group (iii) was also slower compared to control [Group (i) or Group (ii)], before reaching a plateau at 30 minutes of reperfusion (Fig 6.6). During the first 10 min of reperfusion, the HR recovery was significantly ($p<0.05$) worse in Group (iii) compared to Group (i). However, with time, the recovery of HR eventually converged and at the end of reperfusion, there was no difference in the recovery between the groups.

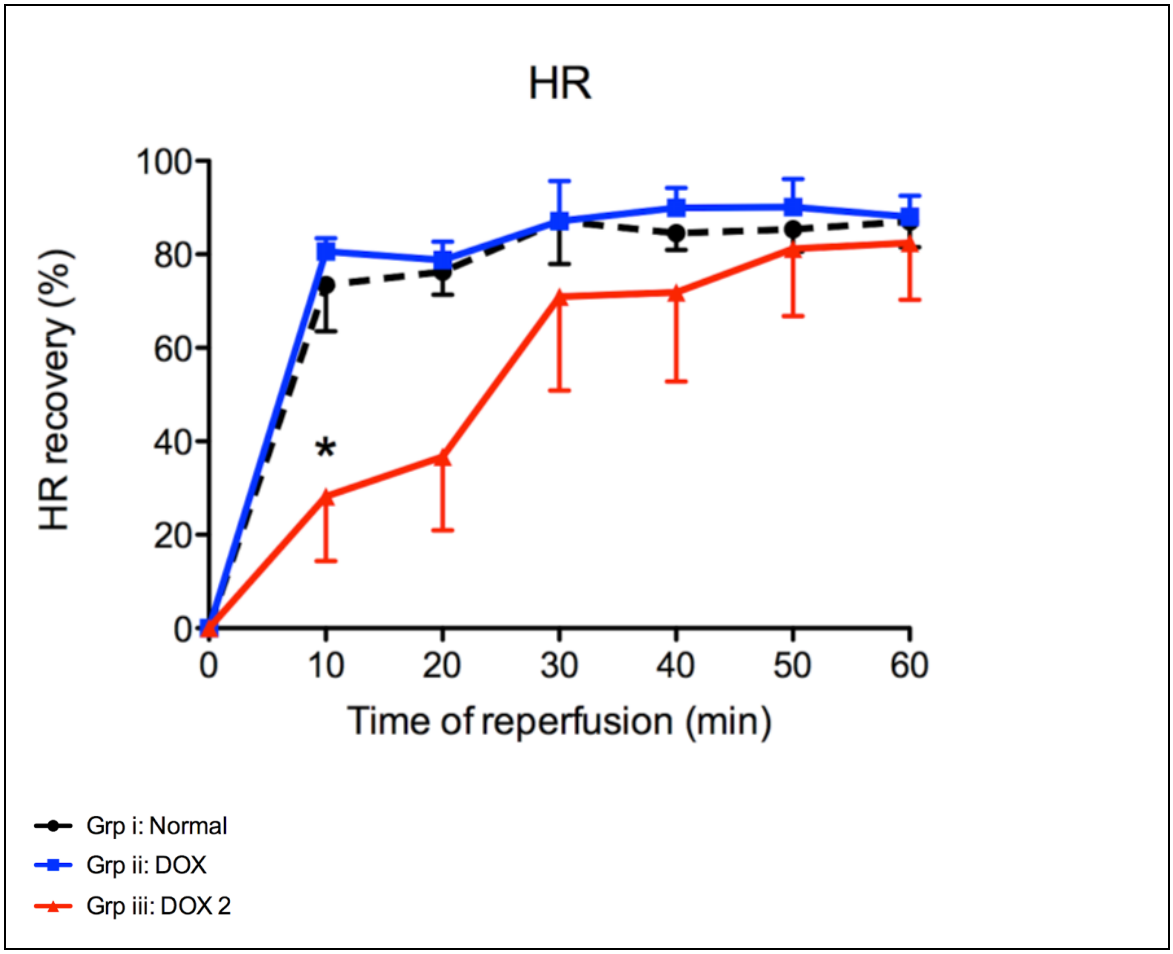


Figure 6.6 Recovery profile of HR in normal hearts during reperfusion after 30 min global ischaemia without and with addition of doxycycline to the perfusate . * $p<0.05$

Group i: Control normal hearts, n=5; Group ii: Normal hearts perfused with 100 µM doxycycline during the last 10 min of aerobic perfusion, n=6; Group iii: Normal hearts perfused with 100 µM doxycycline for 10 min before and 10 min after ischaemia, n=5.

Recovery of CF, too, was worse in Group (iii) compared to Group (i), especially within the first 10 min of reperfusion, eventually converging to equivalent recovery at the end of reperfusion (Fig 6.7).

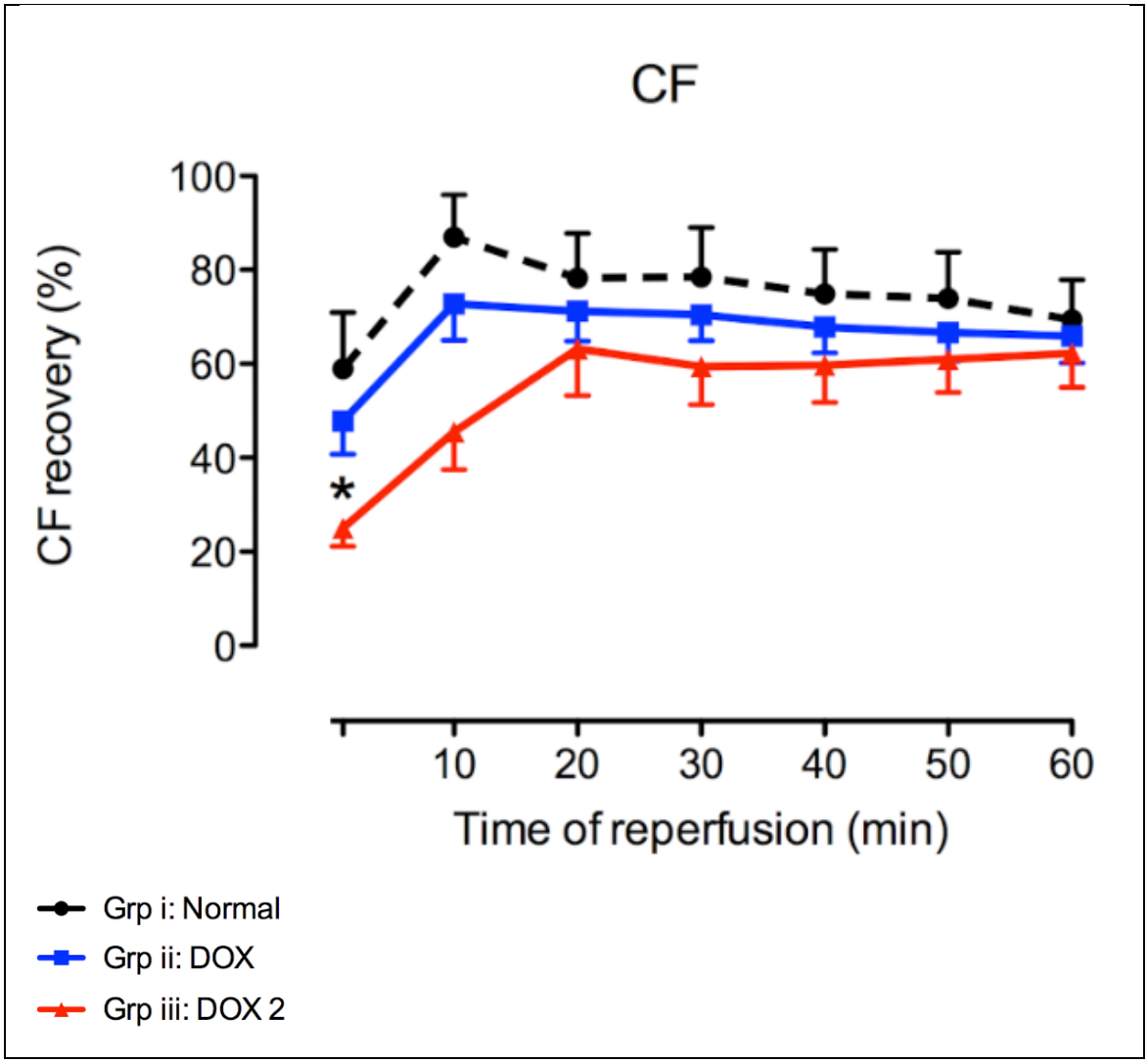


Figure 6.7 Recovery profile of CF in normal hearts during reperfusion after 30 min global ischaemia without and with addition of doxycycline to the perfusate. The first CF measurement was taken during the first min of reperfusion immediately after global ischaemia. * p<0.05

Group i: Control normal hearts, n=5; Group ii: Normal hearts perfused with 100 µM doxycycline during the last 10 min of aerobic perfusion, n=6; Group iii: Normal hearts perfused with 100 µM doxycycline for 10 min before and 10 min after ischaemia, n=5.

As for LVEDP, there was no difference in LVEDP between all 3 groups throughout the 60 min reperfusion period (Fig 6.8).

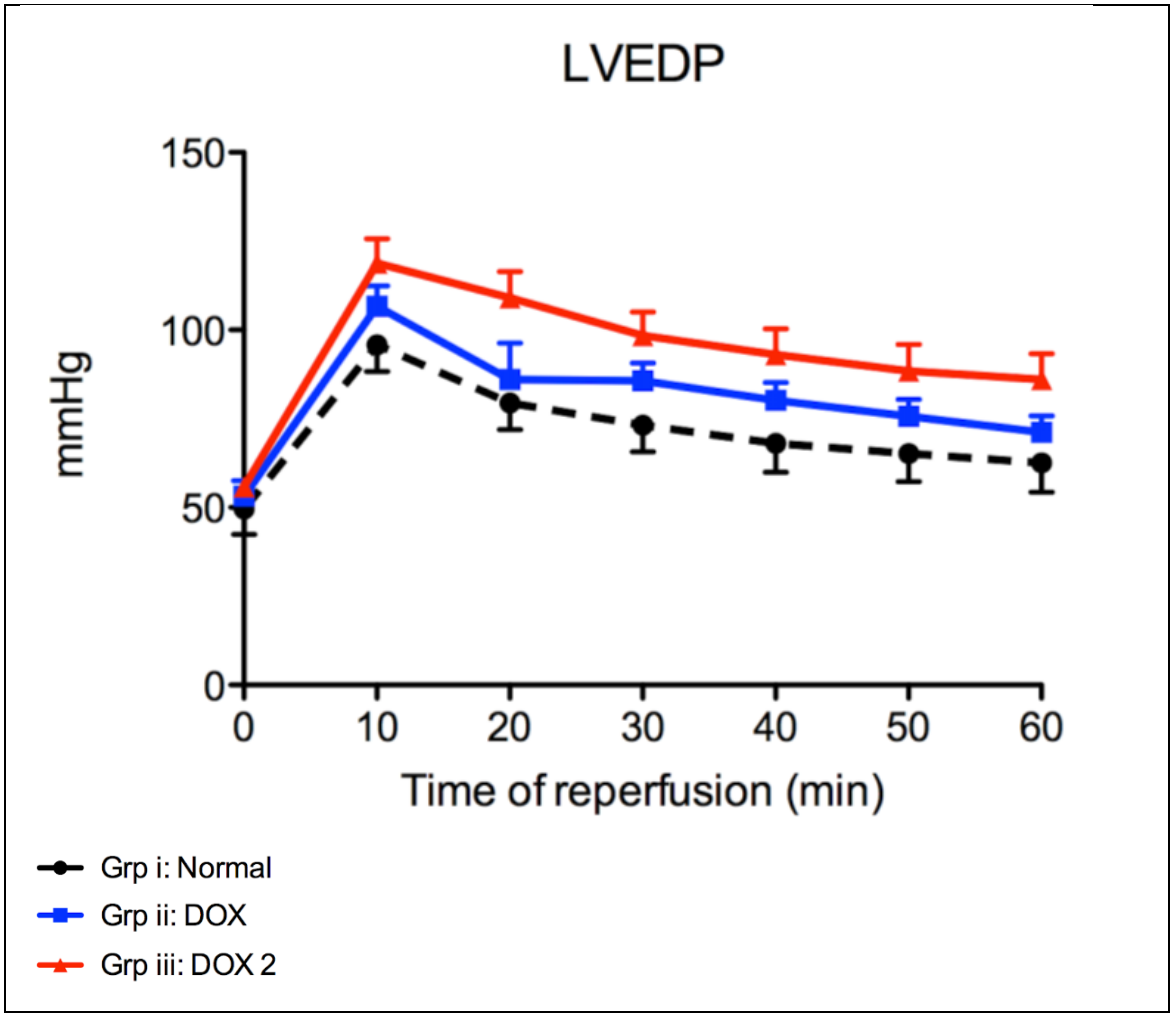


Figure 6.8 Recovery profile of LVEDP in normal hearts during reperfusion after 30 min global ischaemia.

Group i: Control normal hearts, n=5; Group ii: Normal hearts perfused with 100 µM doxycycline during the last 10 min of aerobic perfusion, n=6; Group iii: Normal hearts perfused with 100 µM doxycycline for 10 min before and 10 min after ischaemia, n=5.

As the above data showed, doxycycline, in our study, did not result in any beneficial effects, especially in improving LVDP recovery during reperfusion. In fact, recovery of LVDP was worse when doxycycline was added to the perfusate before and after ischaemia. We therefore decided not to proceed with using doxycycline as the MMP2 inhibitor in infarcted hearts as we did not anticipate any improvement in cardioprotection of the infarcted hearts.

6.3.2 Effect of 1,10-phenanthroline in hearts subjected to acute ischaemia-reperfusion injury

The addition of 100 μM 1,10-phenanthroline to the Krebs-Henseleit perfusate solution significantly changed the physiological parameters during equilibration aerobic perfusion of the infarcted hearts when infused into the hearts (Fig 6.9). The parameters were measured after 10 min of aerobic equilibration (before starting the infusion of 1,10-phenanthroline) and again after 10 min of infusion of 1,10-phenanthroline (before the start of global ischaemia).

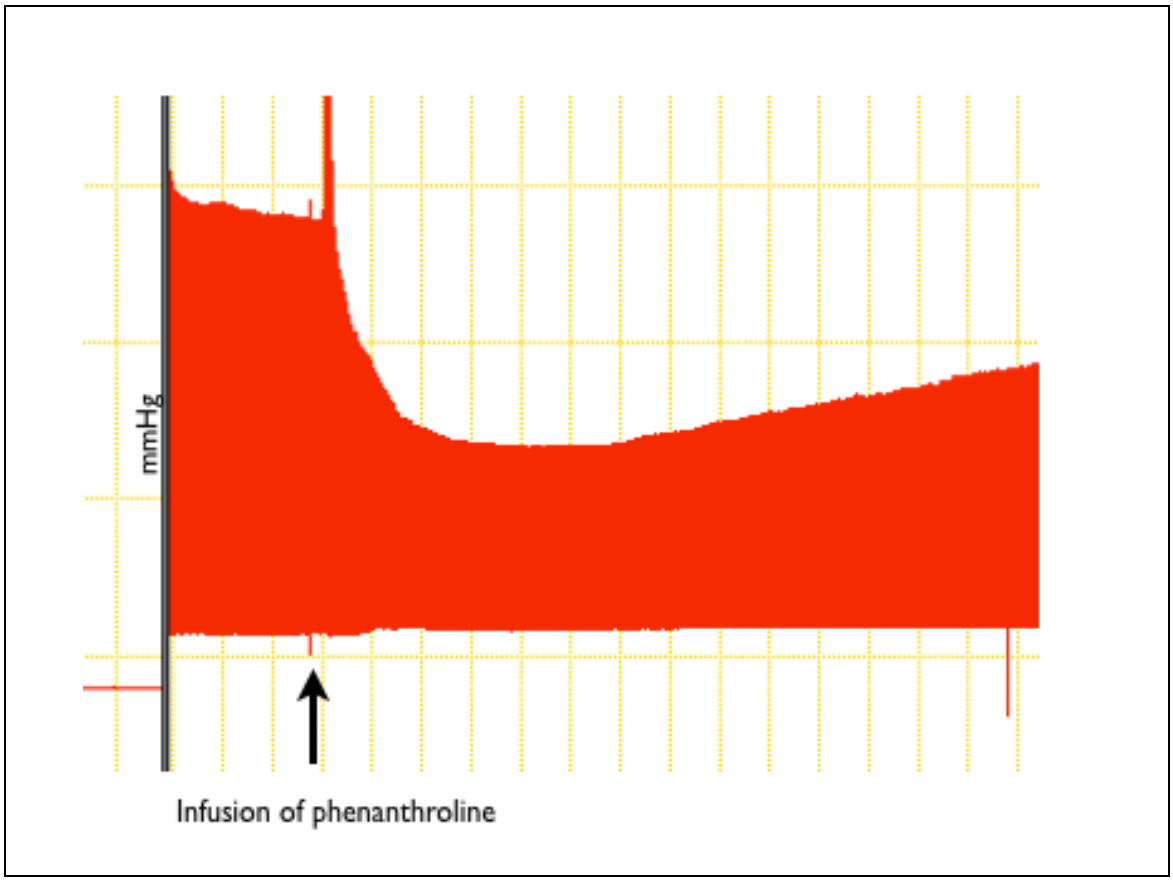


Figure 6.9 Representative recording taken from LabChart during Langendorff perfusion, with addition of 1,10-phenanthroline.

Recording taken from LabChart during Langendorff perfusion, showing the decrease in LV pressure measured at the onset of the infusion of 1,10-phenanthroline, as marked by the arrow.

Thus, LVDP decreased significantly ($p=0.02$) from a mean of 127±12 mmHg to 86±5 mmHg, the heart rate was decreased significantly ($p=0.0005$) from 285±13 beats/min to 173±13 and LVEDP was significantly ($p=0.009$) increased (Fig 6.10).

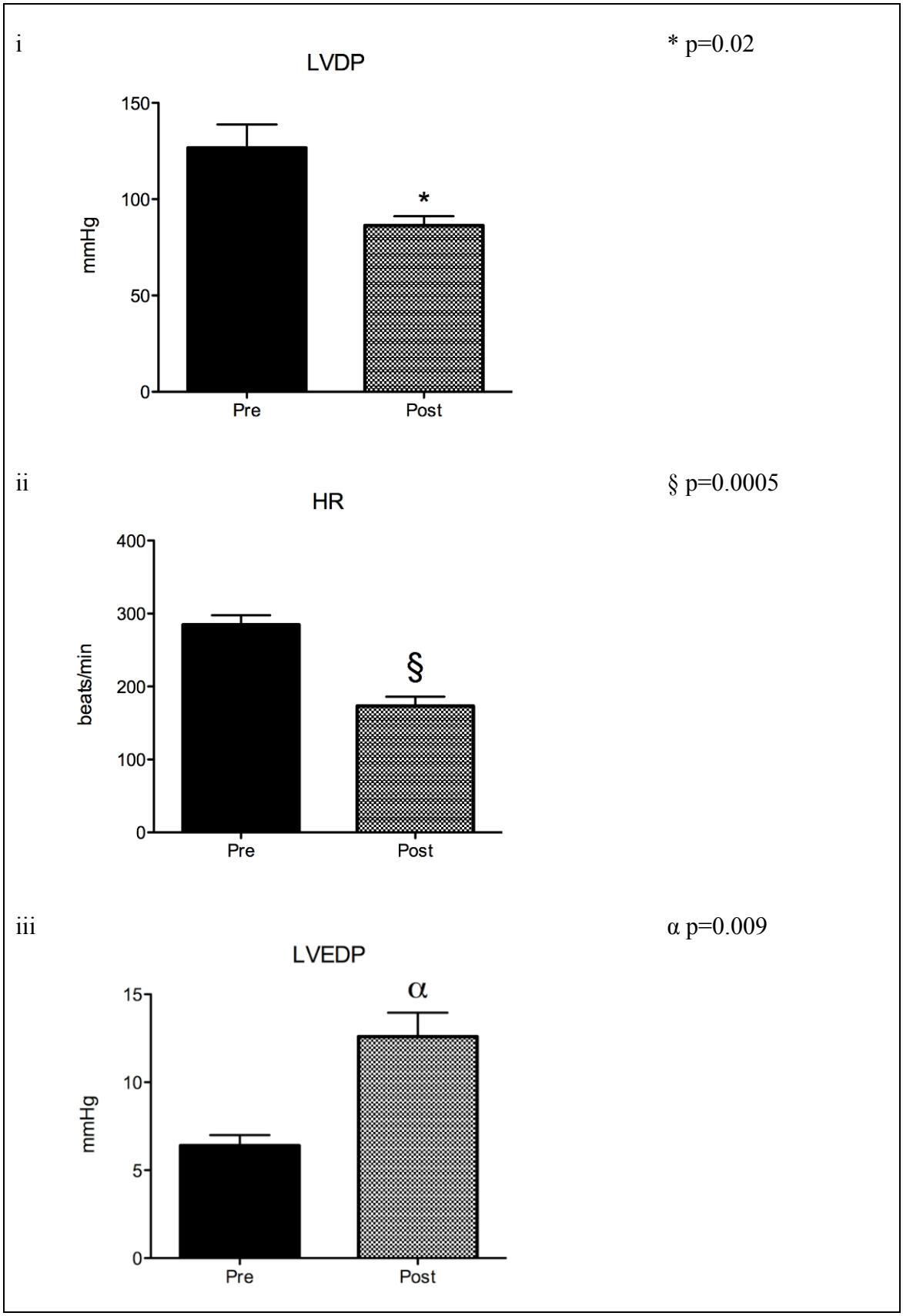


Figure 6.10 Changes in (i) LVDP, (ii) HR and (iii) LVEDP during isolated Langendorff perfusion before and after infusion of 1,10-phenanthroline, during aerobic equilibration, n=5.

With 1,10-phenanthroline treatment before and after ischemia in both normal (Group ii) and infarcted (Group iv) hearts, there was rapid recovery of LVDP, which plateaued by 20 min of reperfusion (Fig 6.11).

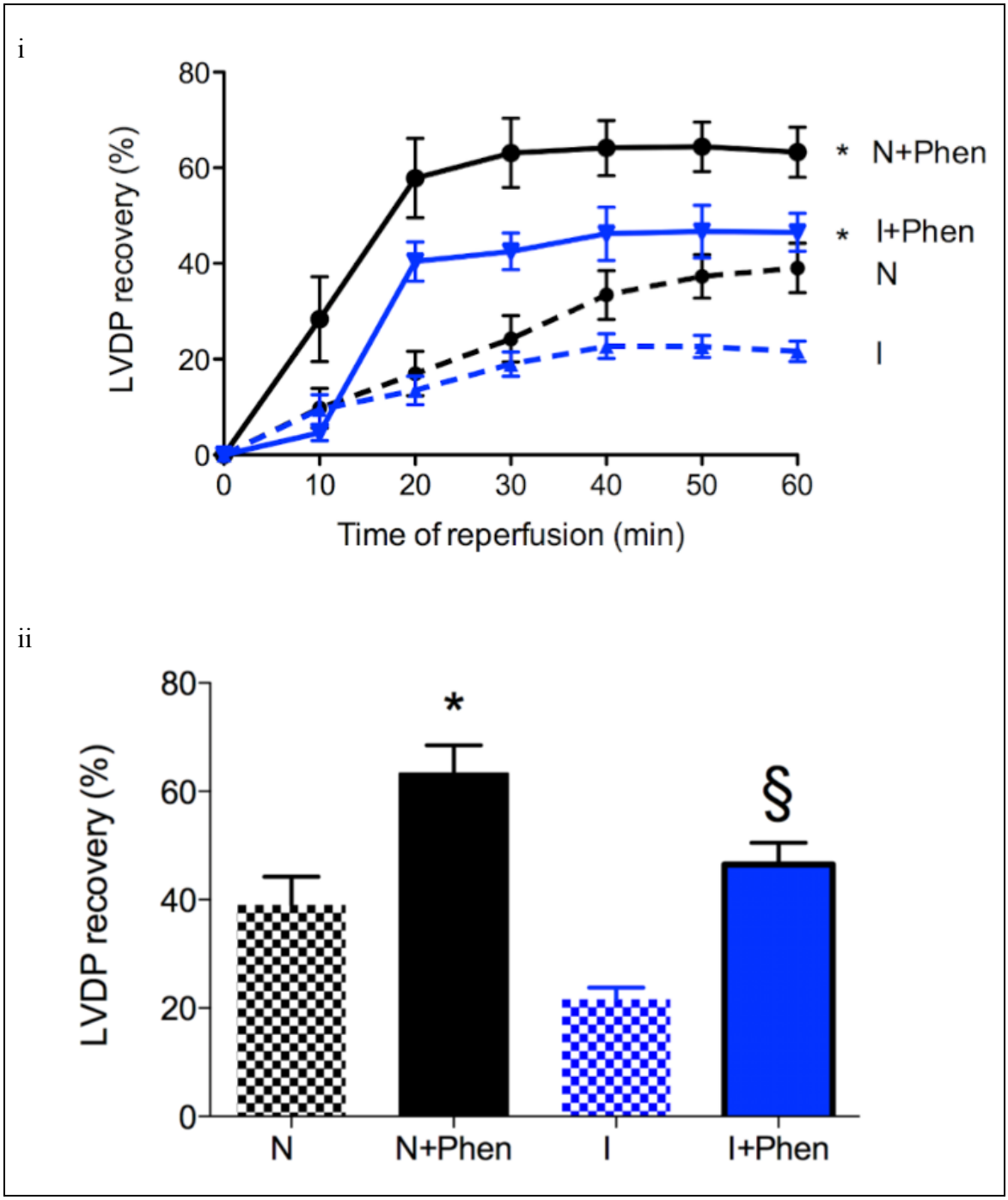


Figure 6.11 Functional recovery (LVDP) in normal and infarcted hearts, with and without 1,10-phenanthroline.

(i) Recovery profiles of LVDP and (ii) final LVDP recovery in normal (N) and infarcted (I) hearts, with and without 1,10-phenanthroline (Phen). n=5 N, n=6 N+Phen, n=6 I and n=5 I+Phen. * p=0.05 N vs N+Phen, § p=0.0002 I vs I+Phen

Comparison of these 1,10-phenanthroline treated hearts with the equivalent normal (Group i) and infarcted (Group iii) hearts (as described in Chapter 4) showed that 1,10-phenanthroline treatment significantly ($p<0.05$) improved recovery in both groups (Fig 6.11i). The final recovery of LVDP achieved in normal hearts and infarcted hearts treated with 1,10-phenanthroline was 63±5% and 47±4% respectively, which was significantly higher compared to the equivalent hearts perfused without MMP inhibition ($p=0.01$ in normal hearts and $p=0.0002$ in infarcted hearts). Interestingly, the final recovery of infarcted hearts treated with 1,10-phenanthroline was similar to untreated normal hearts by 60 minutes of reperfusion (Fig 6.11ii) but 1,10-phenanthroline treatment increased the rate of recovery (after the 1,10-phenanthroline perfusion).

6.3.3 Effect of 444285, a specific MMP2/MMP9 inhibitor, in infarcted hearts subjected to acute ischaemia-reperfusion injury

With the addition of 444285 to the perfusate, both 100 nM and 150 nM significantly decreased the HR compared to prior to the addition of the drug (100 nM, $p=0.002$ and 150 nM, $p=0.005$) (Fig 6.12i). All 3 concentrations of 444285 did not alter LVDP or LVEDP of the hearts during aerobic perfusion (Fig 6.12ii and 6.12iii). Unfortunately, as parallel heart perfusions were carried out, it was not logistically feasible to measure coronary flow before and after infusion of 444285. However, comparison of coronary flow at each concentration of 444285 with coronary flow in control infarcted hearts (as described in Chapter 4) showed that coronary flow in control infarcted hearts without any addition of 444285 was significantly ($p<0.0001$) lower compared to when 50 nM, 100 nM or 150 nM of 444285 was added to the perfusate during the last 10 min of aerobic perfusion (8.7±0.5 mls/min vs 13.4±1 mls/min, 16±0.6 mls/min and 14±1 mls/min, respectively) (Fig 6.13). At 100 nM, the CF was also significantly ($p<0.0001$) higher compared to CF when infarcted hearts were perfused with 100 μM of 1,10-phenanthroline.

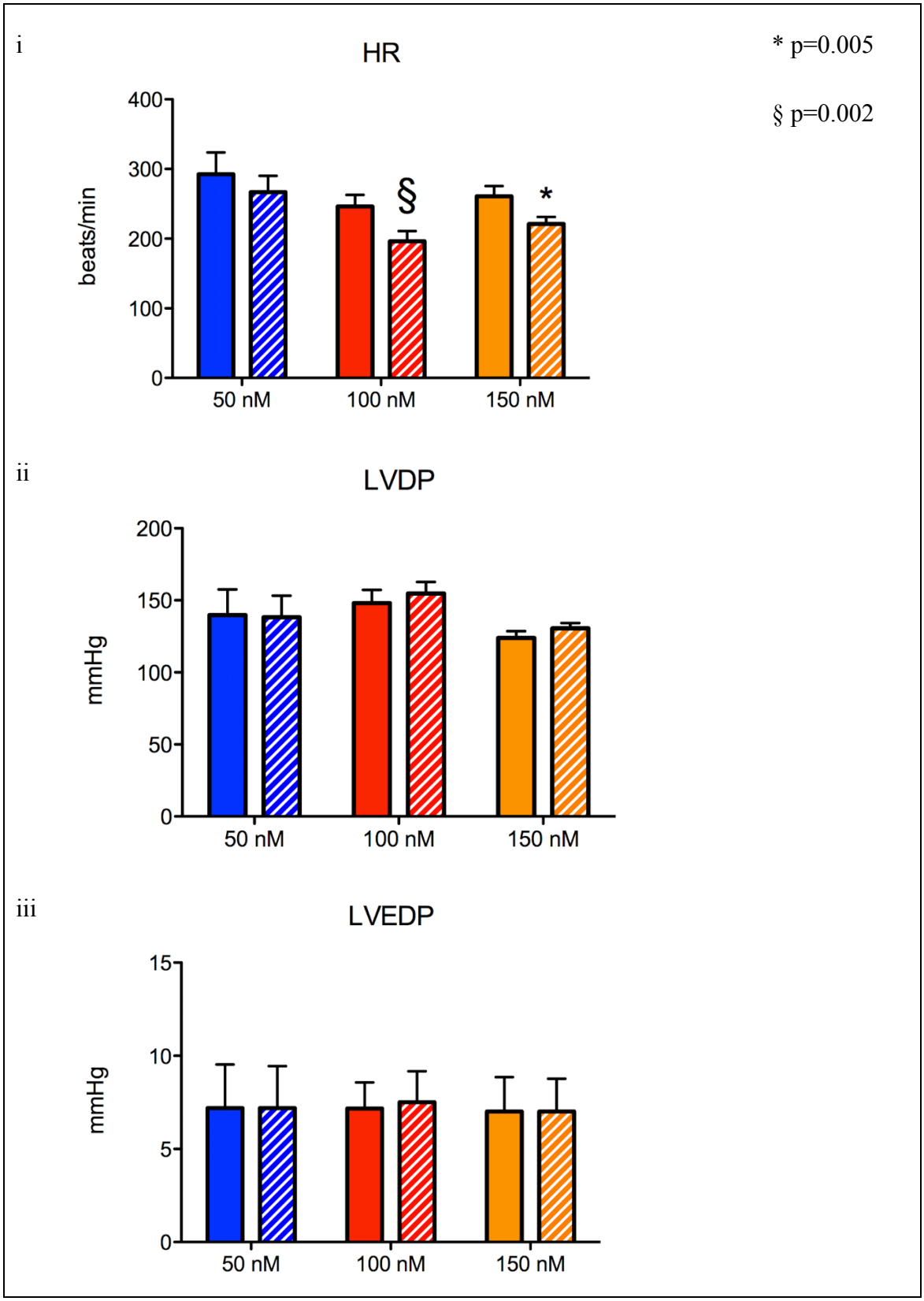


Figure 6.12 Graphs showing the changes in (i) HR, (ii) LVDP and (iii) LVEDP before and after infusion of different concentration of 444285. Solid bar graph – pre-infusion, Clear, striped bar graph – post-infusion. n=5 in 50 nM, n=6 in 100 nM and n=6 in 150 nM

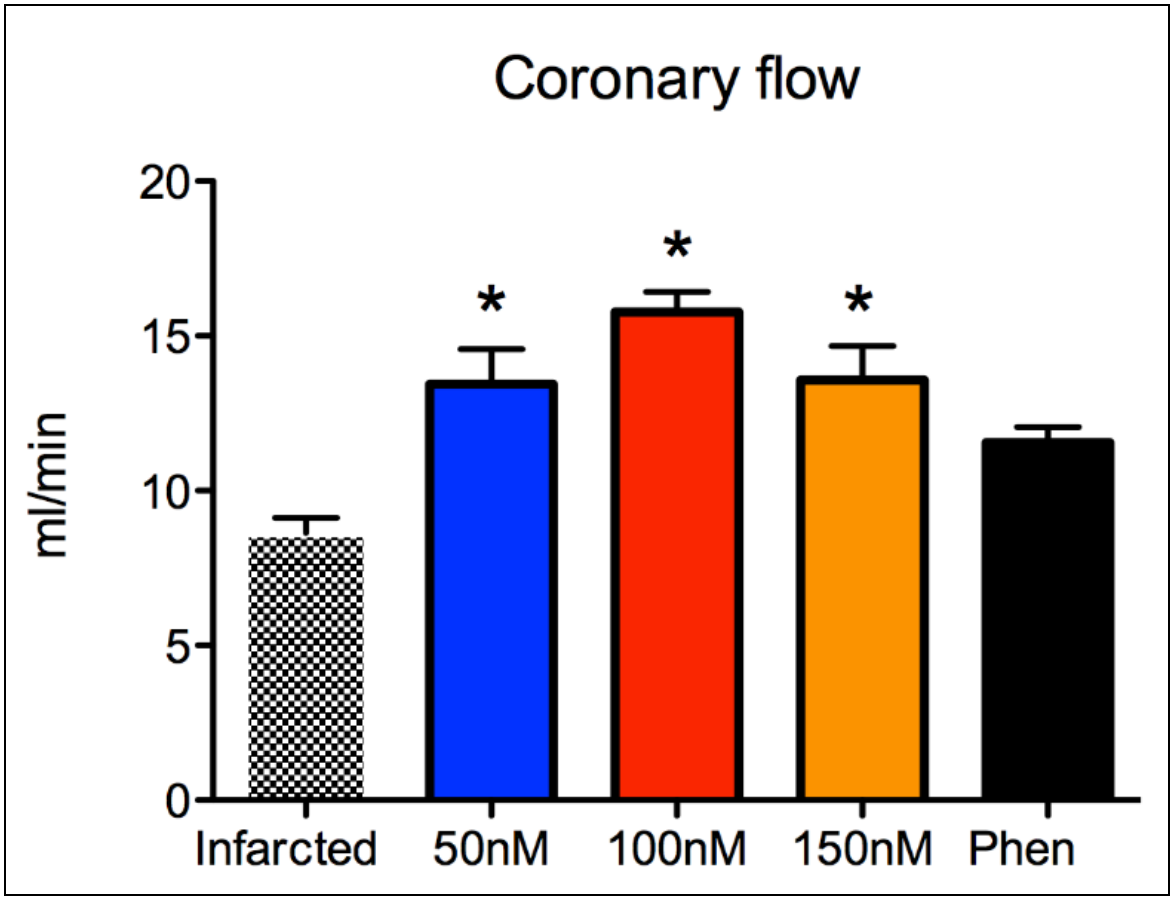


Figure 6.13 Graph showing coronary flow measured after 20 min of aerobic perfusion in infarcted hearts and coronary flow measured in infarcted hearts after 10 min of perfusion with the addition of 50 nM (n=5), 100 nM (n=6), 150 nM (n=6) of 444285 and 100 µM of 1,10-phenanthroline (n=5), respectively. * p<0.0001

During ischaemia, there was no difference in the contracture parameters between infarcted hearts perfused with or without 444285 (Table 6.2).

Table 6.2 Contracture parameters (time to contracture, peak and time to peak) in infarcted, and infarcted hearts perfused with addition of 444285 at 50 nM, 100 nM and 150 nM.

	Infarct (n=5)	50 (n=5)	100 (n=6)	150 (n=6)
Time to contracture (min)	13.5±0.4	14.8±0.4	14±1.3	14.3±0.6 (p=0.74)
Peak (mmHg)	61±8	52±9	61±8	51±6 (p=0.33)
Time to peak (min)	17.7±0.7	20.4±0.5	19.2±1.1	18.5±0.7 (p=0.16)

The recovery of LVDP during the first 30 min of reperfusion between different concentrations of 444285 was similar to each other and to infarcted hearts without 444285 (Fig 6.14i). However, after 40 min of reperfusion, the recovery of LVDP in infarcted hearts perfused with and without 444285 started to diverge. The recovery profile of the groups was significantly ($p<0.0001$) different compared to equivalent hearts without 444285. The final recovery achieved in the 100 nM group at 32±2% was significantly ($p=0.02$) higher (22±2%) than infarcted hearts perfused without 444285 (Fig 6.13ii).

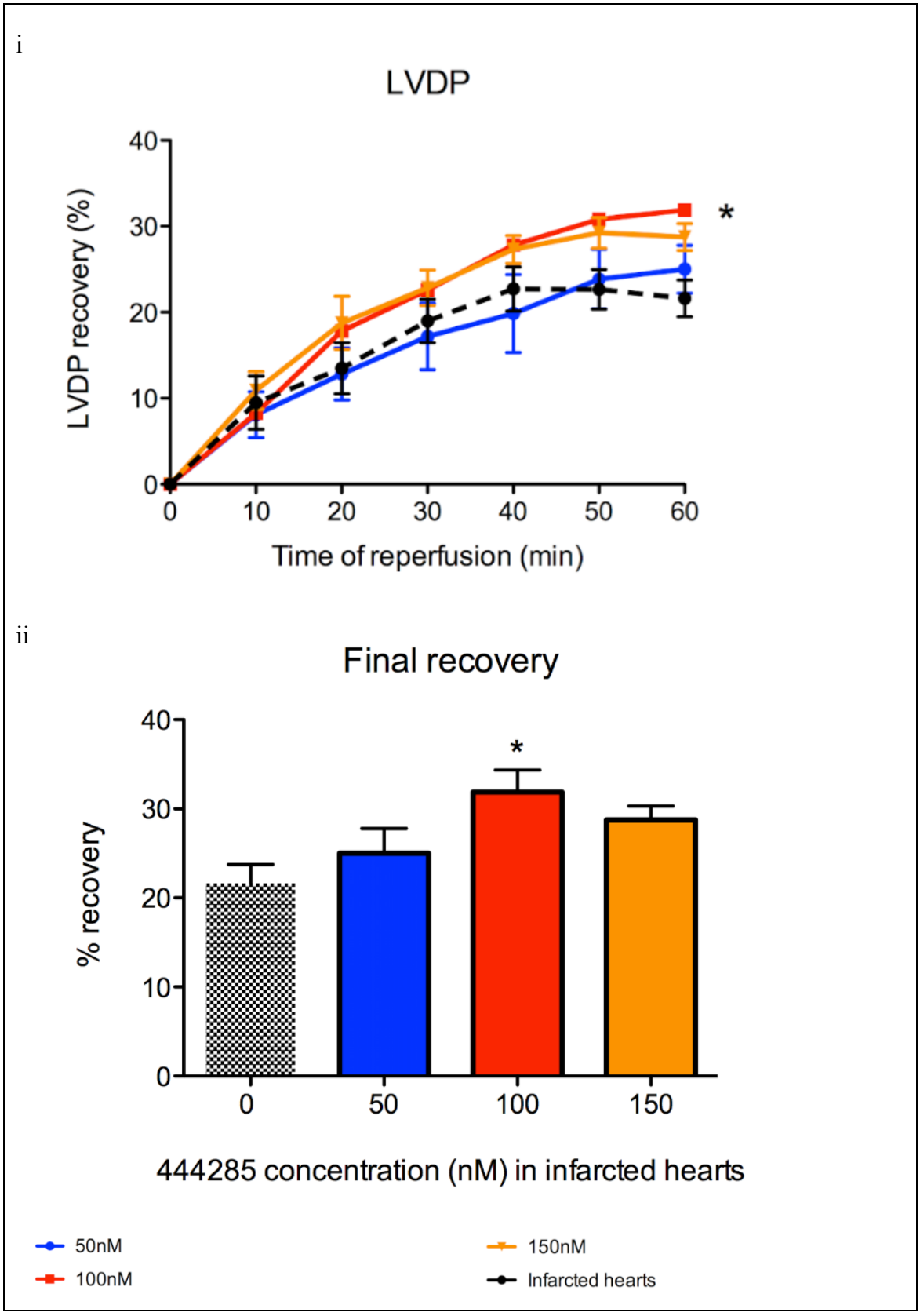


Figure 6.14 Recovery profile of (i) LVDP and (ii) final LVDP recovery in infarcted hearts perfused with 50 nM (n=5), 100 nM (n=6) and 150 nM (n=6) of 444285 compared to infarcted hearts (n=6) from studies described in Chapter 4. *p<0.05

As for the HR, the groups perfused with 444285 had a gradual recovery before reaching a plateau at 30 min of reperfusion, which contrasted slightly with the infarcted hearts perfused without 444285, where the peak maximal recovery was reached at 20 min of reperfusion, before plateauing to a lower value (Fig 6.15).

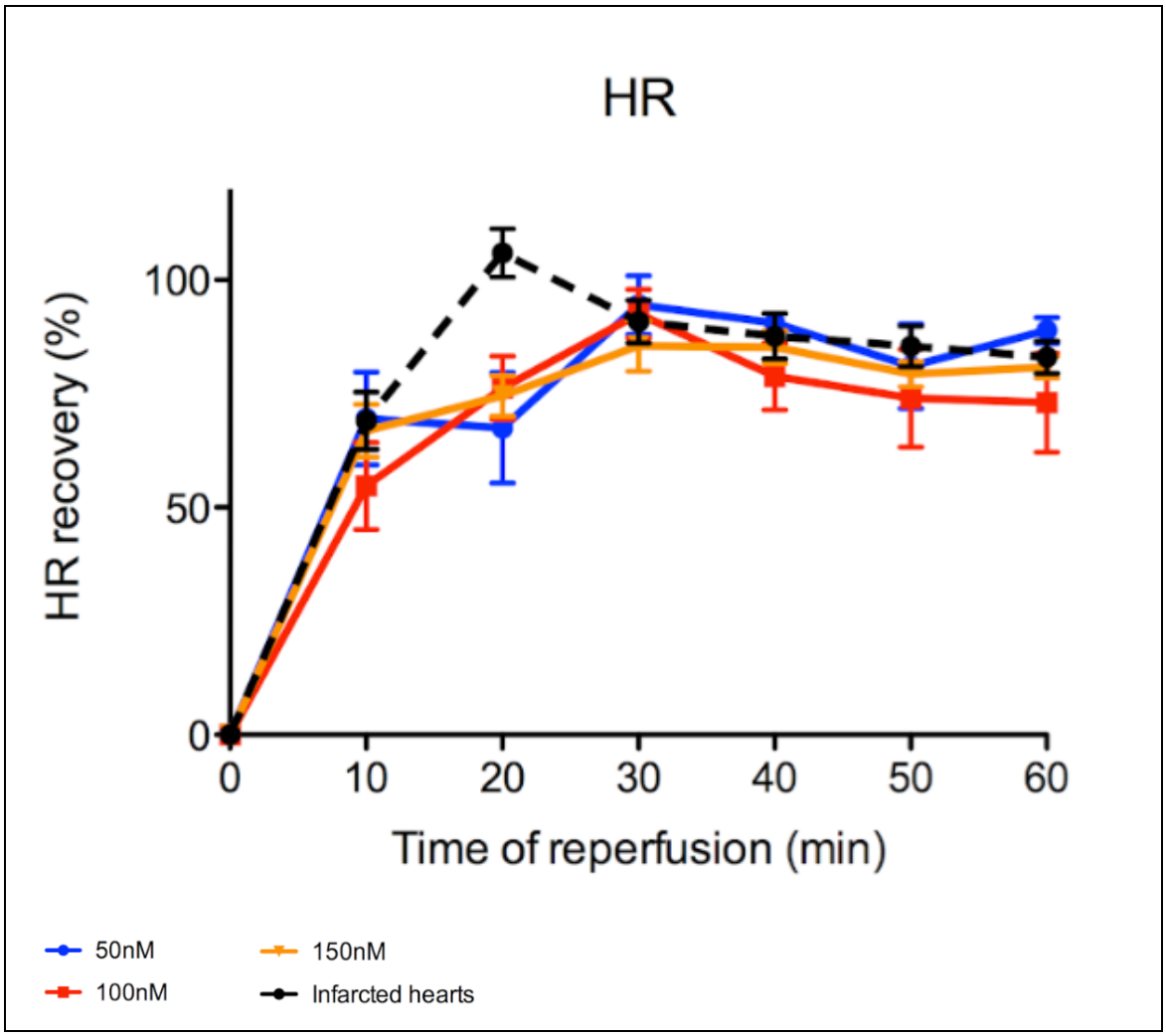


Figure 6.15 Recovery of HR in infarcted hearts perfused with 50 nM (n=5), 100 nM (n=6) and 150 nM (n=6) of 444285 compared to infarcted hearts (n=6) previously perfused without 444285 in Chapter 4.

The profile of recovery of LVEDP and CF of all groups followed a very similar course (Fig 6.16).

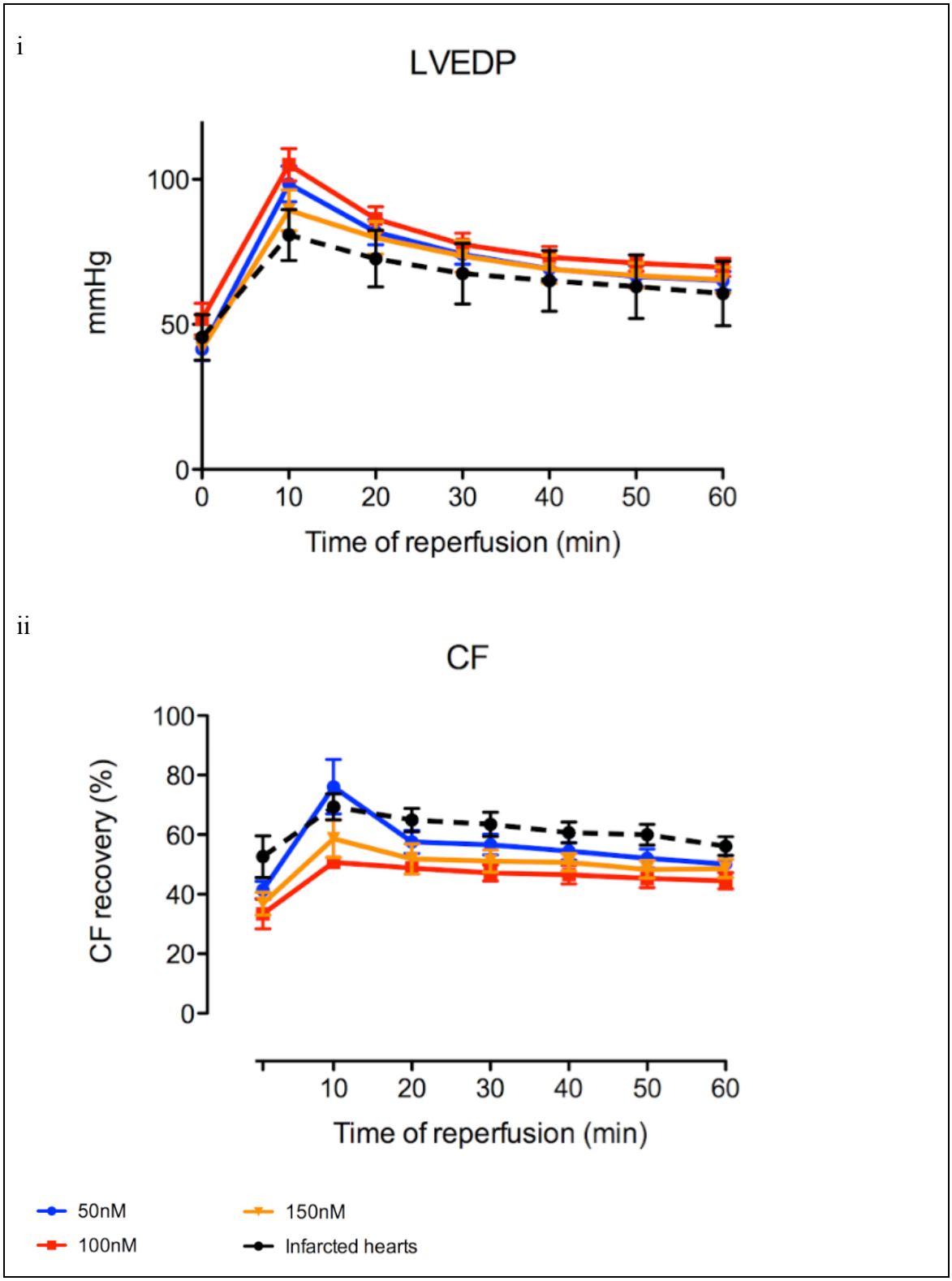


Figure 6.16 The recovery profile of (i) LVEDP and (ii) CF in infarcted hearts perfused with 50 nM (n=5), 100 nM (n=6) and 150 nM (n=6) of 444285 compared with infarcted hearts (n=6) without 444285 described in Chapter 4. The first CF measurement was taken during the first min of reperfusion after global ischaemia.

6.3.4 Effect of 444285 on myocardial MMP2 activity in infarcted hearts subjected to acute ischaemia-reperfusion

In Group 1 (infarcted hearts without addition of 444285), MMP2 activity peaked after 30 min of global ischaemia before gradually decreasing back to its baseline value.

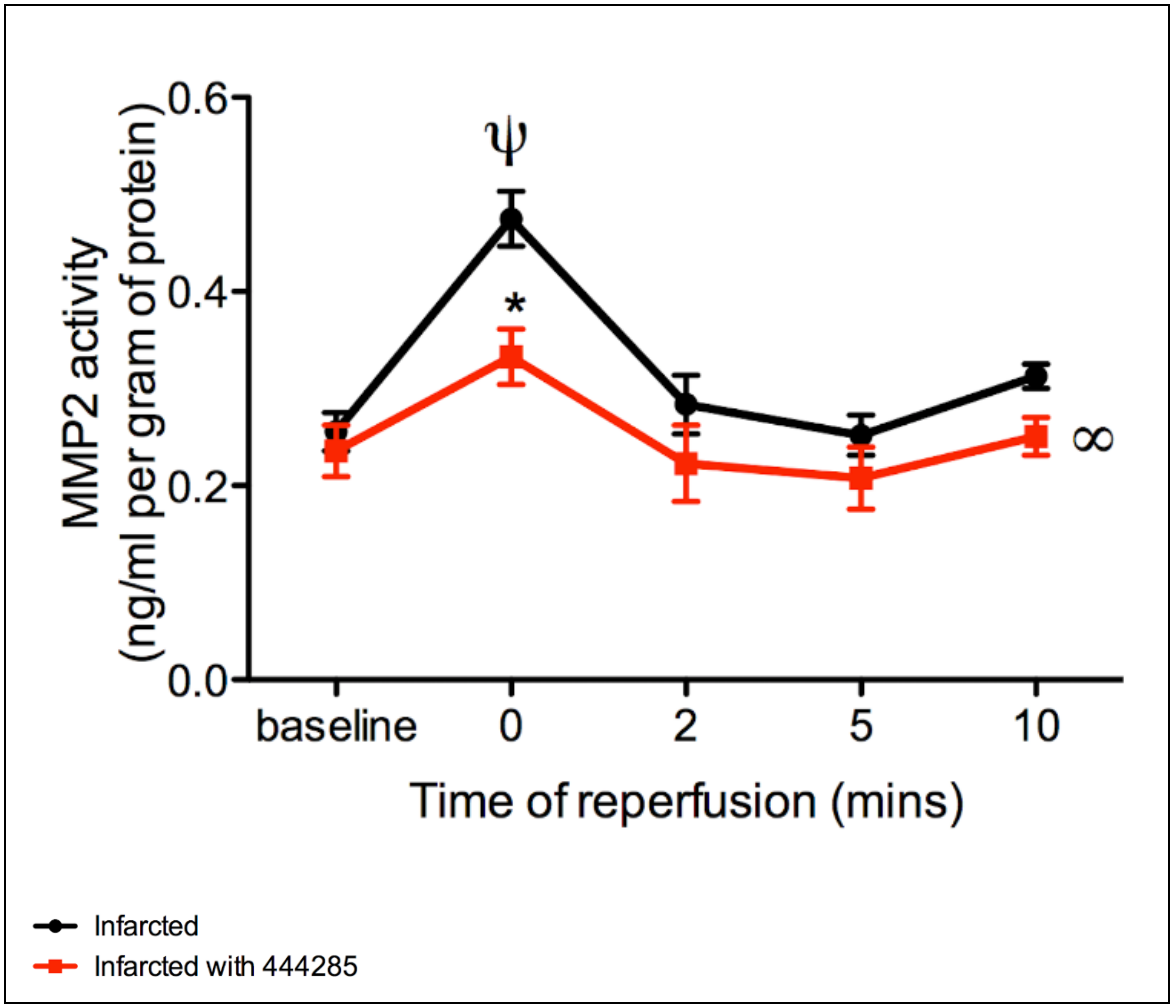


Figure 6.17 MMP2 activity in infarcted hearts perfused with or without 100 nM of 444285 at baseline aerobic perfusion, after global ischaemia (0 min) and at 2, 5 and 10 min of reperfusion (n=4 in each group at each time point). ψ p<0.0001 vs other sampling times in infarcted hearts without 444285, ∗ p=0.02 vs MMP2 activity in infarcted hearts without 444285 immediately after global ischaemia, ∞ p=0.047 vs MMP2 activity in infarcted hearts without 444285 at 10 min reperfusion.

The peak of MMP2 activity immediately after global ischaemia was significantly ($p<0.0001$) higher compared to the other time points assessed (Fig 6.17). When 444285 was added to the perfusate 10 min before and after ischaemia (Group 2), the trend of MMP2 activity was similar to hearts in Group 1; there was a smaller peak immediately

after ischaemia, but this was not significantly higher compared to the other time points, unlike in Group 1. However, MMP2 activity immediately after global ischaemia in Group 2 was significantly (p=0.02) lower compared to the activity in hearts in Group 1. After 10 min of reperfusion, MMP2 activity in infarcted tissues perfused with 444285 was also significantly (p=0.047) lower compared to hearts perfused without 444285.

6.4 Discussion

There have been a number of studies investigating the effects of MMP inhibitors on isolated perfused rat hearts subjected to ischaemia-reperfusion [35, 36, 38, 79, 128]. In an experimental in-vitro setting, Cheung and co-workers demonstrated that there was improved recovery of mechanical function (using rate-pressure product as the index of function) of isolated hearts subjected to acute ischaemia-reperfusion when doxycycline (at different concentrations ranging from 10 μM to 100 μM) was added to the perfusate [35]. In contrast, we were unable to confirm these results with a doxycycline concentration of 100 μM (the optimal dose established by Cheung and colleagues [35]), with no significant improvement in the recovery of LV function of isolated hearts in our studies (Section 6.3.1). There are a few fundamental differences in the 2 studies, which may explain the results. In our study, we used a global ischaemic duration of 30 min compared to a 20 min duration used in Cheung's study. An additional 10 min may be sufficient to alter the capacity of the heart to recover, as previously shown in Chapter 4, Section 4.3. The index of mechanical function and recovery was different between the 2 studies; we use % recovery of LVDP compared to its baseline pre-ischaemic values, whereas the rate-pressure product was the index used by Cheung and co-workers. The mode of delivery of doxycycline differed in that constant pressure was used in our studies, in contrast to constant flow. This may be crucial, especially in the early reperfusion phase when coronary flow was usually depressed. Collectively, these subtle differences may explain the results obtained in our study. In a rabbit model of isolated heart perfusion, Donato and colleagues looked at the role of MMP2 in ischaemic post-conditioning on the heart [132]. They found that ischaemic post-conditioning decreased infarct size in the heart, with inhibition of MMP2 activity. This decrease in infarct size was duplicated when doxycycline, at a concentration of 50 μM, was administered to

hearts without post-conditioning, and the extent of reduction of infarct size was similar to ischaemic post-conditioning. However, in their experiments, although zymographic analysis showed that there was decreased MMP2 activity in the coronary effluent and ventricular tissues within 2 min of reperfusion in hearts that underwent post-conditioning protocol and hearts reperfused with doxycyline, there was no observed improvement in the functional recovery (LVDP) of the heart. Hence, in vitro evidence of enzyme activity inhibition does not necessarily translate to functional recovery. These contradictory observations may be due to several unresolved issues regarding tetracyclines as MMP inhibitors. Although doxycycline is the most potent MMP inhibitor in this group, generally they are still relatively weak and broad, non-specific MMP inhibitors. The inhibitory constants of doxycycline are in the micromolar range compared to other potent inhibitors with inhibitory constants in nanomolar range [133].

Nonetheless, the profile of recovery of LVDP in normal hearts perfused with doxycycline showed that recovery was still increasing at the end of the observed 60 min reperfusion period (Fig 6.5). It is possible that the method of delivery of doxycycline in our model resulted in a delayed recovery, and perhaps with longer reperfusion time i.e 120 min, the recovery achieved may be equivalent to studies carried out by Cheung and associates. It is difficult to speculate. A longer reperfusion time also has its disadvantages, as the isolated heart will start to deteriorate [85]. It is unlikely that they would show a beneficial effect, even with longer reperfusion period.

In contrast, inhibition with another non-specific and reversible MMP inhibitor, 1,10-phenanthroline, resulted in a rapid and much improved recovery in LVDP. In an effort to comply with the 3R principles of animal research (to reduce the number of animals used), dosage studies to determine the optimal dose for 1,10-phenanthroline were not carried out. The concentration selected was based on previous studies, which had demonstrated that 100 μM was an effective concentration for MMP inhibition [35, 38]. Our studies were similar to other studies [35]; infusion of perfusate containing 1,10-phenanthroline significantly altered function. Cheung and colleagues also reported that, with infusion of 100 μM 1,10-phenanthroline, the rate-pressure product (used as an index of mechanical function in their study) was significantly depressed, but recovered once infusion of 1,10-phenanthroline was stopped. Our study showed that LVDP was reduced and LVEDP increased, but once the infusion was stopped after 10 min, there

was rapid recovery of LVDP, as shown by the steep gradient of the recovery curve compared to equivalent controls. The effect on LVDP could be secondary to its Ca^{2+}-chelating actions. However, as it is a reversible inhibition, once the infusion was stopped, its effects were washed out with perfusion using regular KHB. The data suggested that even a short, reversible inhibition of MMP protected the hearts and resulted in improved functional recovery.

Perfusion studies with a selective and irreversible inhibitor, 444285, further reinforced the role of MMP2 in cardiac dysfunction in infarcted hearts subjected to further global ischaemia. There was a dose-dependent improvement in recovery, with an optimal concentration of 100 nM. As this inhibitor has not been used in other isolated Langendorff heart perfusion studies (to our knowledge), the effects of the inhibitor on the physiological parameters were considered. The only striking difference was the significantly higher coronary flow in hearts perfused with 444285, at all 3 concentrations tested. This is not surprising given the evidence available showing the vasoactive role of MMP2. The secretory profiles of MMP2 and MMP9 in microvasculature differed from macrovasculature [134]. In cultures of bovine coronary arterial endothelial cells, active MMP2 decreased nitric oxide (NO) release in these cells in response to agonist stimulation [135]. Additionally, selective inhibition of MMP2 resulted in vasodilation of rat mesenteric arteries [136]. Our data also showed that selective and irreversible inhibition of MMP2, even for a short duration of 10 min, was sufficient to inhibit MMP2-induced vasoconstriction. MMP2 activity assay provided evidence that improved functional recovery was associated with significant inhibition of myocardial MMP2 activity. The improvement in recovery profile and final recovery was not as good as MMP inhibition with σ-phenanthroline. This may be due to the fact that 444285 does not inhibit MMP2 activation by MT1-MMP. MT1-MMP has been shown to be activated during ischaemia-reperfusion in the heart [42]; MT1-MMP in turn activates MMP2 [11]. Hence, compared to 1,10-phenanthroline, the extent of MMP2 inhibition by 444285 may be less.

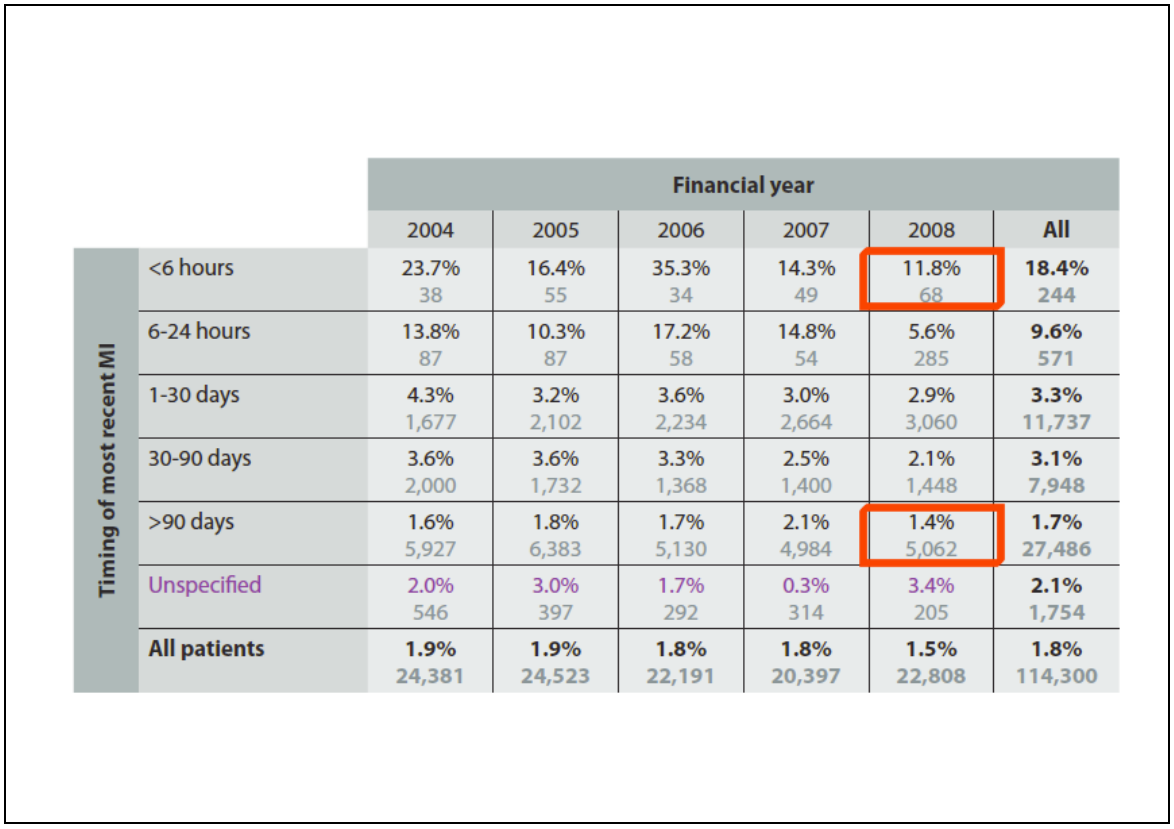
In summary, the data presented here showed the link between improvement in mechanical function of isolated hearts (normal and infarcted) subjected to acute ischaemia-reperfusion and molecular evidence of MMP2 inhibition, when the hearts were perfused with MMP2 inhibitors. MMP2 inhibition may be potential novel targets

or as adjuncts in cardioprotection of hearts during cardiac surgery. Cardiac surgery is a unique situation where global ischaemia and reperfusion of the heart is carried out in a controlled environment. This provides an excellent opportunity to introduce drugs at specific times, such as at the onset of ischaemia or at the start of reperfusion, that may alter enzyme or protein effects that are activated by ischaemia-reperfusion and may be detrimental to the recovery of function of the hearts. It also allows short-term treatment rather than longer-term treatment with drugs that may have potential side effects when taken over a longer period of time. Besides, as previously discussed in Chapter 1, enzymes such as MMPs may have beneficial effects, all depending on the timing from injury. This forms the basis of our investigation in the subsequent and final chapter. We want to investigate the effect of short-term MMP2 inhibition, used in conjunction with cardioplegia solution, in protecting the heart as would happen during cardiac surgery. We selected 1,10-phenanthroline as an addition to a standard cardioplegia solution, the St Thomas' Hospital cardioplegia solution, as Section 6.3.2 and 6.3.3 showed that there was greater extent of recovery in the infarcted hearts treated with 1,10-phenanthroline compared to 444285.

7 THE INFLUENCE OF CARDIOPLEGIA (ST THOMAS HOSPITAL SOLUTION) WITH AND WITHOUT MMP INHIBITOR ON THE RECOVERY OF INFARCTED HEARTS

7.1 Introduction

The demographics of patients undergoing cardiac surgery have changed with time [70]. The mean age of patients having isolated coronary artery bypass graft surgery (CABG) increased slightly from 63 years in 2000 to 66 years in 2008, but the proportion of >75 years undergoing surgery more than doubled from 10% in 1999 to 25% in 2008. Crude mortality of patients over the age of 80 years undergoing isolated CABG was about five times higher compared to those under the age of 56 in 2008. The proportion of patients with significant coronary artery disease affecting the left main stem (LMS) of the left coronary artery has also doubled between 2001 and 2006, and these patients have double the mortality rate. There is also a changing trend in patients needing urgent or emergency CABG, increasing from 29.8% in 2001 to 32.9% in 2008. The proportion of patients having surgery within a month of suffering from ACS increased from 19% in 2004 to 34% in 2008. The mortality in patients having CABG within 6 hours of suffering an MI was 11.8%, but there was a stepwise decrease in operative mortality with increasing interval between MI and surgery, with mortality approaching that of elective surgery if MI was >90 days prior to surgery (Fig 7.1). The change in this trend probably reflects the change in management of patients presenting with ACS, as discussed in Chapter 1. Often, patients with LMS disease are also patients requiring urgent or emergency surgery. These factors, when considered together, raise the risk profile of patients undergoing CABG. There is now an even greater need for improved protection of the heart during surgery to improve the outcome.



Timing of most recent MI	Financial year					
	2004	2005	2006	2007	2008	All
<6 hours	23.7% 38	16.4% 55	35.3% 34	14.3% 49	11.8% 68	**18.4%** 244
6-24 hours	13.8% 87	10.3% 87	17.2% 58	14.8% 54	5.6% 285	**9.6%** 571
1-30 days	4.3% 1,677	3.2% 2,102	3.6% 2,234	3.0% 2,664	2.9% 3,060	**3.3%** 11,737
30-90 days	3.6% 2,000	3.6% 1,732	3.3% 1,368	2.5% 1,400	2.1% 1,448	**3.1%** 7,948
>90 days	1.6% 5,927	1.8% 6,383	1.7% 5,130	2.1% 4,984	1.4% 5,062	**1.7%** 27,486
Unspecified	2.0% 546	3.0% 397	1.7% 292	0.3% 314	3.4% 205	**2.1%** 1,754
All patients	**1.9%** 24,381	**1.9%** 24,523	**1.8%** 22,191	**1.8%** 20,397	**1.5%** 22,808	**1.8%** 114,300

Figure 7.1 Data from the 6th National Adult Cardiac Surgical Database Report 2008.

The crude mortality rate according to the timing of the most recent MI. Highlighted boxes emphasised the decrease in mortality the longer the interval from MI to surgery.

As previously mentioned (Chapter 1, Section 1.4), St Thomas' Hospital cardioplegic solution is one of the most commonly used cardioplegic solutions during cardiac surgery and protects the heart reasonably well during cardiac surgery; however, it is not without certain disadvantages. Ongoing research into improving cardioprotection includes improving or supplementing St Thomas' Hospital cardioplegic solution [55], other types of cardioplegia [57, 137] and also alternative means of cardioprotection besides chemical arrest of the heart, for example intermittent fibrillation of the heart [56]. MMP2 inhibition may play a role as a supplement to St Thomas' Hospital cardioplegia solution due to a number of reasons. Firstly, inhibition of MMP2 may reduce its intracellular action on structural and functional proteins during acute ischaemia-reperfusion injury. MMP2 was shown to be activated by peroxynitrite, a substrate generated from oxidative stress injury to the heart [79]; thus, inhibition of MMP2 may reduce the detrimental effects of oxidative stress. Lastly, appropriate inhibition of MMP2 may improve protection of

the coronary vasculature by its effects on the endothelium. It has been shown to have both vasocontrictive and vasodilatory effects [13]. MMP2 can vasoconstrict arteries by acting on endothelin-1 (ET-1), producing a potent vasoconstrictor peptide [138] or by cleavage of calcitonin gene-related peptide (CGRP), which ameliorated the vasodilatory effects of CGRP [136]. On the other hand, its vasodilatory effect is mediated by the inhibition of Ca^{2+} entry mechanism [13].

Regarding the role of MMP2 in cardiac surgery, cardiopulmonary bypass and cardioplegia, there are various small studies that suggested a role for MMPs in causing cardiac dysfunction associated with cardiopulmonary bypass. It is well-established that cardiopulmonary bypass (CPB) circuits invoke a systemic inflammatory response, with enhanced release and activation of neutrophils. Increased circulating neutrophils mediate the secretion of MMP9 into the circulatory system post-CPB, with significantly increased plasma levels and activity by 2 to 6 hours after weaning from CPB [139]. Using a microdialysis technique combined with fluoroscopy, overall MMP activity was found to increase significantly with initiation of CPB, remained at the same level when cross-clamp (ischaemic time) was started, decreased back to almost baseline with diastolic arrest of the heart, before increasing again with reperfusion and weaning from CPB [140]. The decrease in MMP activity during mid-CPB time was thought to be secondary to the cardioprotective strategy, where hypothermic cardioplegia was used. During and after CPB, there was a distinct pattern of increased MMP activity; MMP8 rapidly increased and then decreased after weaning from CPB, and there was similar increase in MMP13 and MMP9 and return to baseline within 6 hours of weaning from CPB. In contrast, MMP2 increased between 6 and 24 hours after separation from CPB [141]. The amount of MMP2 in atrial tissue at reperfusion correlated negatively with the function of the heart as measured by LV stroke work index (LVSWI) [142]. The higher the amount of MMP2 present, the poorer the function of the heart.

I previously established the temporal profile of MMP2 (Chapter 5), with MMP2 activity in infarcted myocardium being significantly higher compared to normal or sham hearts. If these hearts are subsequently subjected to other distinct events (i.e. cardiopulmonary bypass and/or global no-flow ischaemia), that have been shown to independently activate MMP2 and was detrimental to the mechanical function of the heart, these events could have synergistic effects that will enhance the injury imposed on an already

stressed heart. MMP2 inhibition may indeed be a new strategy to protect a vulnerable myocardium perioperatively during cardiac surgery.

MMPs have a wide range of biological actions, sometimes causing opposite effects of biological processes such as triggering cell apoptosis as well as cell proliferation and activating and inactivating cytokines and chemokines [122]. As Barbara Fingleton puts it "the optimal use of MMP inhibitors hinges on identifying the deleterious MMP activity and targeting that while sparing any non-contributory or beneficial effects" [123]. Hence, the timing and duration of MMP inhibition affects outcome. Pharmacological inhibition of MMP2 and MMP9 decreased the risk of cardiac rupture post-MI, but continued inhibition could be damaging as it prevented healing and ultimate resolution [43, 143].

Hence, in this chapter, we explore the possibilities of short-term MMP2 inhibition as an adjunct to St Thomas' Hospital cardioplegic solution, in improving cardioprotection of the infarcted myocardium. The timing of MMP2 inhibition was also explored using different isolated heart perfusion protocols.

7.2 Methods

Adult, male Wistar rats (250-350 g) (n=6/group) were used in these experiments. The animals were first subjected to surgical ligation of the LAD artery as described in Chapter 2, Section 2.1, and allowed to recover for 7 days. After 7 days, the hearts were harvested and perfused in an isolated Langendorff heart perfusion as described in Chapter 2, Section 2.2. During isolated Langendorff perfusion, the hearts were equilibrated for 20 min with aerobic perfusion, before global, normothermic ischaemia was induced by clamping the aortic inflow line for 60 min, followed by 60 min of reperfusion with oxygenated perfusate. St Thomas' Hospital cardioplegic solution (composition in mM: NaCl 11; $MgCl_2$ 16; KCl 16; $CaCl_2$ 1.2 and $NaHCO_3$ 10; pH 7.8 at 37°C) was used to arrest the heart at the start of ischaemia. Preliminary studies were conducted to determine the appropriate ischaemic duration when using cardioplegic protection; the ischaemic period was initially extended to 40 min (multiple infusion of St Thomas' Hospital cardioplegia for 2 min every 20 min). Using 3 infarcted hearts, the recovery of LVDP was about 60% of pre-ischaemic baseline value (Fig 7.2). Thus, I

decided to extend the period of global ischaemia to 60 min (infusion of cardioplegia for 2 min repeated after 30 min).

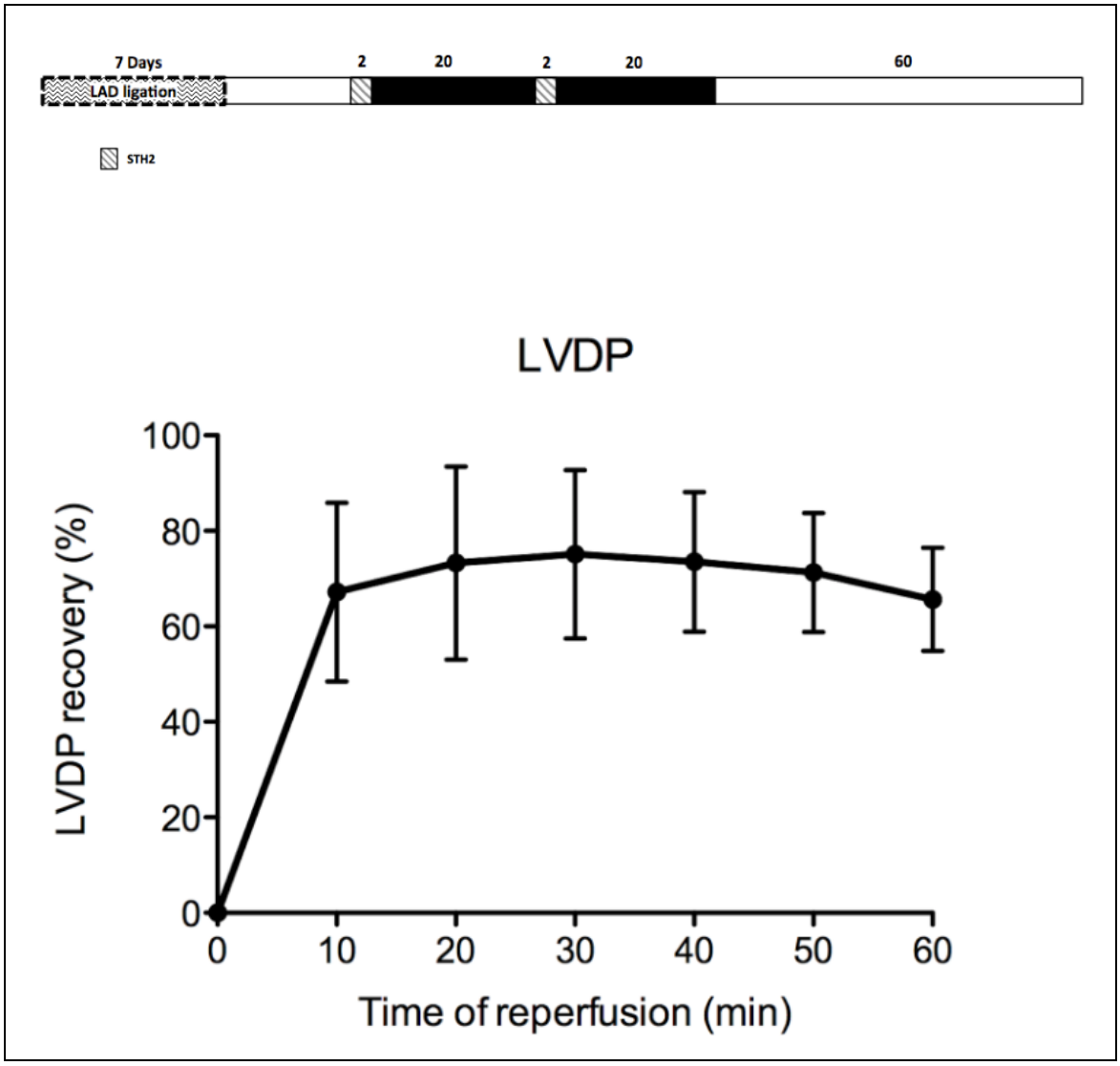


Figure 7.2 Recovery profile in infarcted hearts, arrested with St Thomas' cardioplegia.

(Top insert) Langendorff perfusion protocol of infarcted hearts, with hearts arrested using St Thomas' cardioplegia and 40 min global ischaemia.

(Bottom) LVDP recovery (% pre-ischaemic value) profile in infarcted hearts perfused according to the perfusion protocol depicted in top insert (n=3).

The various protocols and infusion of cardioplegia solution and MMP inhibitor are shown in Fig 7.3. 1,10-phenanthroline (100 μM) was used in these experiments as it was shown in Chapter 6 that it resulted in the best recovery of mechanical function of the infarcted hearts.

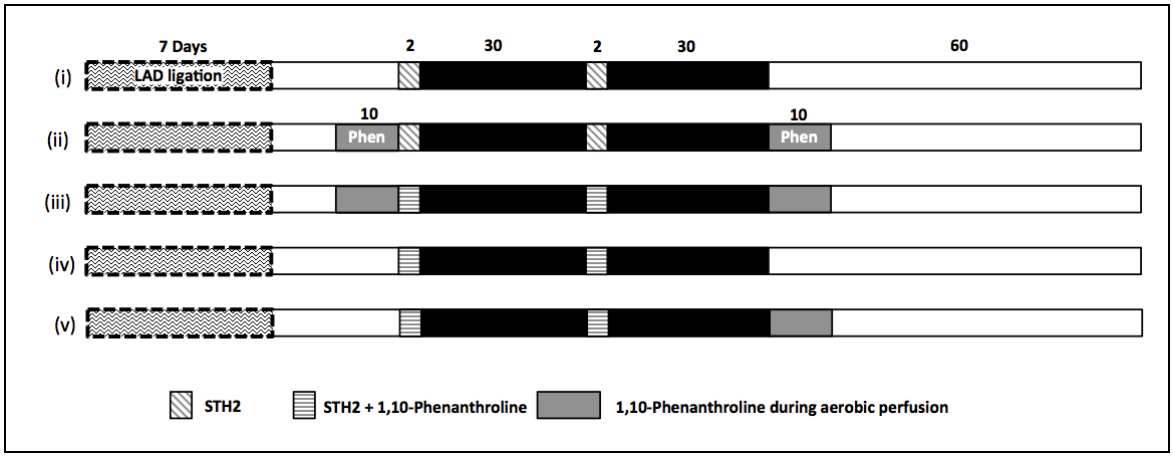


Figure 7.3 Langendorff perfusion protocol in infarcted hearts, with St Thomas' cardioplegia protection (n=6/group).

All hearts arrested with STH2 cardioplegia (i & ii) or STH2+1,10-phenanthroline (iii, iv & v). 1,10-phenanthroline added in addition in (ii), (iii) and (v) as shown.

In Group (i), the hearts were arrested with St Thomas' Hospital cardioplegia solution only. In Group (ii), the hearts were arrested with St Thomas' Hospital solution and 1,10-phenanthroline was added to the KHB perfusate 10 min before and after ischaemia. In Group (iii), the hearts were arrested with St Thomas' Hospital solution plus 1,10-phenanthroline, and 1,10-phenanthroline was included in the KHB perfusate 10 min before and after ischaemia. In Group (iv), the hearts were arrested with St Thomas' Hospital cardioplegia plus 1,10-phenanthroline. In Group (v), the hearts were arrested with St Thomas' Hospital cardioplegia plus 1,10-phenanthroline, and 1,10-phenanthroline was added to the perfusate during the first 10 min of reperfusion. These various combinations of cardioplegia with 1,10-phenanthroline was used to determine if the presence of MMP inhibition during the various phases of ischaemia-reperfusion will impact the recovery of the hearts during reperfusion.

The HR, LVDP and LVEDP were monitored and acquired continuously throughout the protocol. CF was measured every 10 min during the reperfusion period. The recovery of LVDP was expressed as % recovery of its pre-ischaemic value. The recovery profile during reperfusion phase was analysed using the exponential association curve analysis and 2-way repeated measures ANOVA of % LVDP recovery was also carried out. The final LVDP recovery at the end of 60 min reperfusion was compared between the

groups with one-way ANOVA and post-hoc Tukey's test was performed (for multiple comparisons). A value of $p<0.05$ was considered statistically significant.

7.3 Results

7.3.1 Ischaemic contractures

The parameters for ischaemic contracture are shown in Fig 7.4. During ischaemia, the time to onset of contracture for hearts in each group was (i) 32±3.2 min, (ii) 25±1.0 min, (iii) 35±0.2 min, (iv)29±3.0 min and (v)32±2.0 min, respectively (Fig 7.4i). Group (ii) developed contracture significantly ($p=0.02$) earlier than the hearts in Group (iii). The times taken for peak contracture to develop were 49±1.5, 42±1.0, 49±1.0, 45±3.0, and 42±0.8 min, in Groups (i) to (v) respectively (Fig 7.4ii). Groups (ii) and (v) reached peak contracture significantly ($p=0.009$) earlier compared to Group (i). Peak contracture reached in the hearts in each group were 34±5, 50±4, 33±3, 50±6 and 50±4 mmHg, respectively; however, there were no statistically significant differences between the groups (Fig 7.4iii).

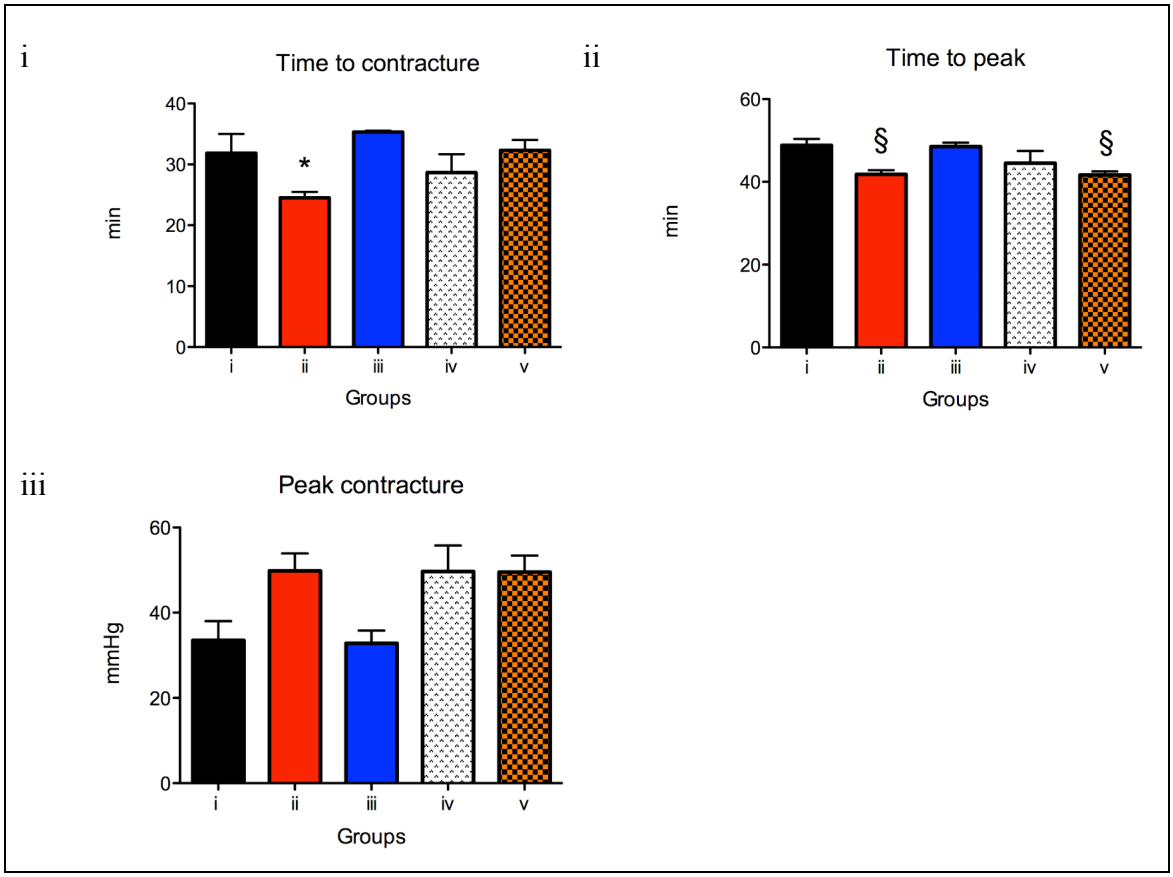


Figure 7.4 Contracture parameters in the each group during ischaemia (n=6/group). *p=0.02, § p=0.009

7.3.2 Haemodynamic recovery

The LVDP recovery profiles of each group of hearts were significantly ($p<0.0001$) different (Fig 7.5). The recovery of LVDP in Groups (i) and (iv) were the most rapid, recovering within the first 10 min and reaching a plateau by 20 min of reperfusion. On the other hand, recovery rates in the first 10 min in Groups (ii) and (iii) were slower before accelerating between 10 and 20 min of reperfusion. This observation was expected, secondary to the effect of 1,10-phenanthroline as previously found in Chapter 6, Section 6.3.2. The recovery in Group (v) was the worst, with a much slower rate throughout reperfusion. At 20 and 30 min of reperfusion, LVDP recovery was significant better in Group (iii) compared to Group (v) (51±10% vs 18±3%, $p<0.01$; 56±8% vs 27±3%, $p<0.05$). The final recovery of LVDP was 47±10%, 45±7%, 57±6%, 39±5% and 36±4% in groups (i), (ii), (iii), (iv) and (v) respectively; these values did not reach statistical significance.

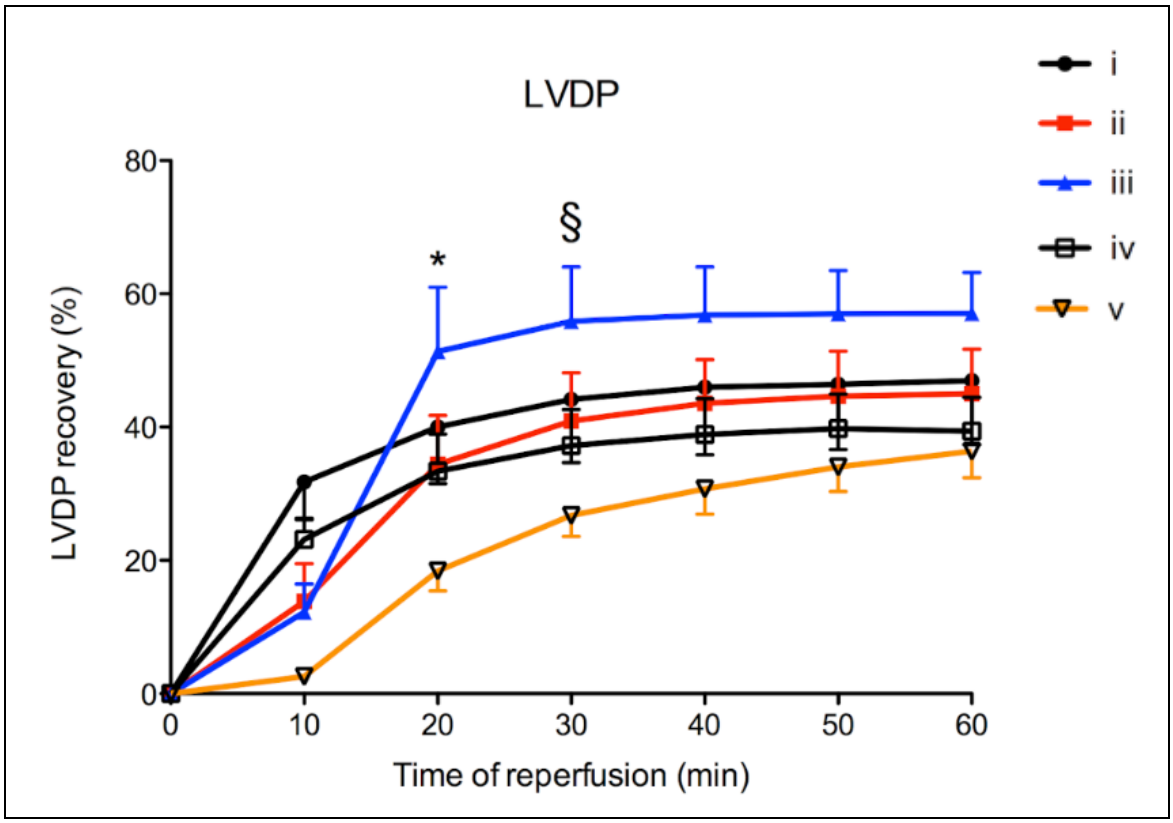


Figure 7.5 Functional recovery in infarcted hearts, with St Thomas' cardioplegia protection (n=6/group).

LVDP recovery profiles in all groups during reperfusion after 60 min of global ischaemia. * p<0.01, § p<0.05

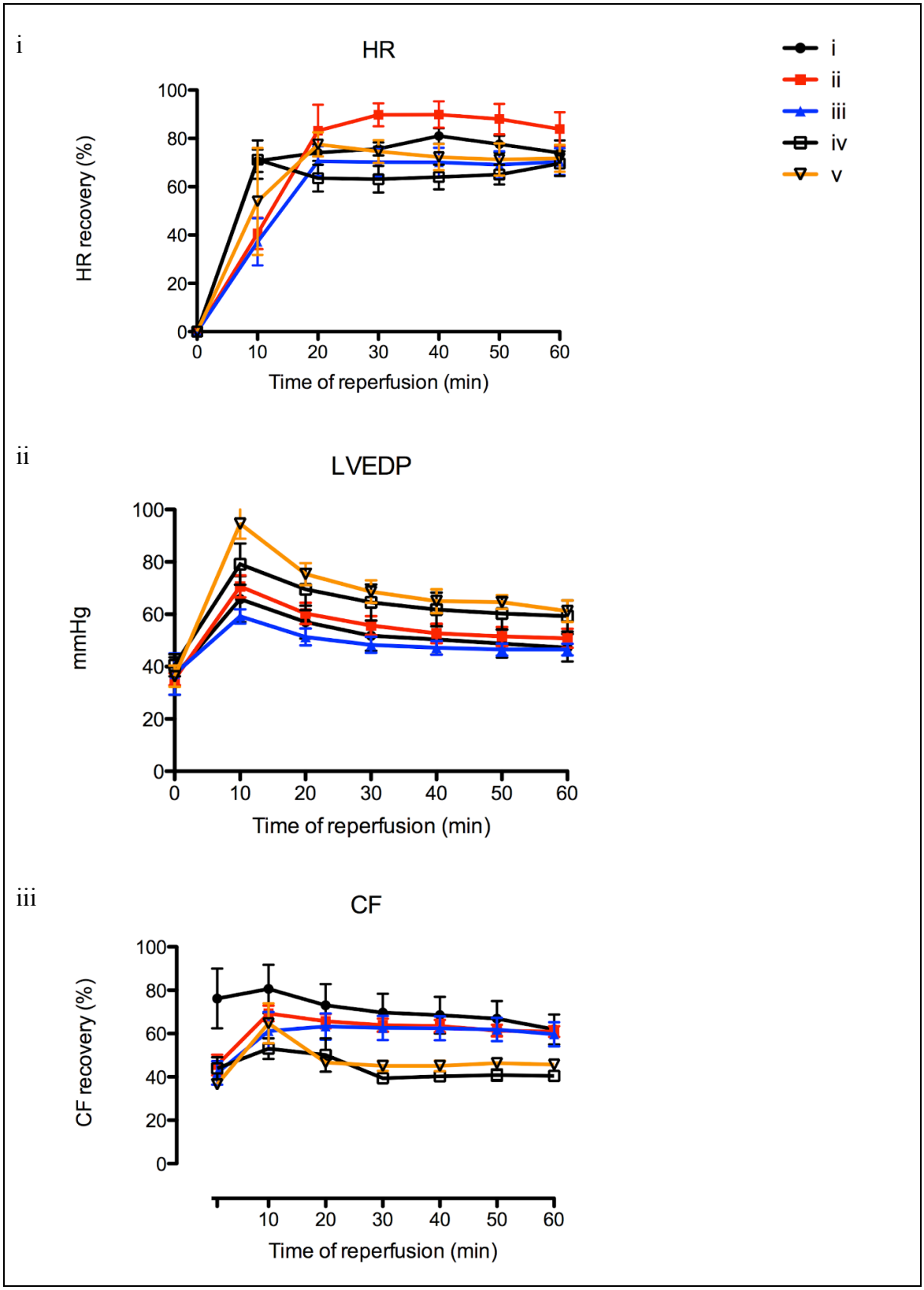


Figure 7.6 Recovery profiles of (i) HR, (ii) LVEDP and (iii) CF of the different groups infarcted hearts during reperfusion after 60 min of global ischaemia (n=6/group). First CF measurement taken during the first min of reperfusion after global ischaemia.

The recovery of HR in the different groups was similar, converging at 60 min of reperfusion to a recovery of between 70% to 80% of the pre-ischaemic value (Fig 7.6i). The recovery of LVEDP in all groups also followed a very similar trend, with increased LVEDP during the first 10 min of reperfusion, which subsequently declined but remained significantly higher than the pre-ischaemic value (Fig 7.6ii). Recovery of CF showed a slightly divergent trend; groups (i) to (iii) were fairly consistent, reaching a final recovery of about 50% of the pre-ischaemic value (Fig 7.6iii). However, Groups (iv) and (v) had a slightly lower final recovery of between 40-45%.

Finally, we also considered the volume of cardioplegia infused in all the groups. The total volume, although slightly higher in Group (iii) at 28.1±3.6 ml, was not significantly different between the groups (Fig 7.7i). The volumes of cardioplegia was further analysed by dividing the total into first and second infusions. For the initial infusion at the onset of ischaemia, the volume of cardioplegia infused was significantly ($p=0.02$) higher in Group (v). However, the volume of second infusion of cardioplegia was significantly ($p=0.003$) higher in Group (iii) at 12.3±2.5 ml.

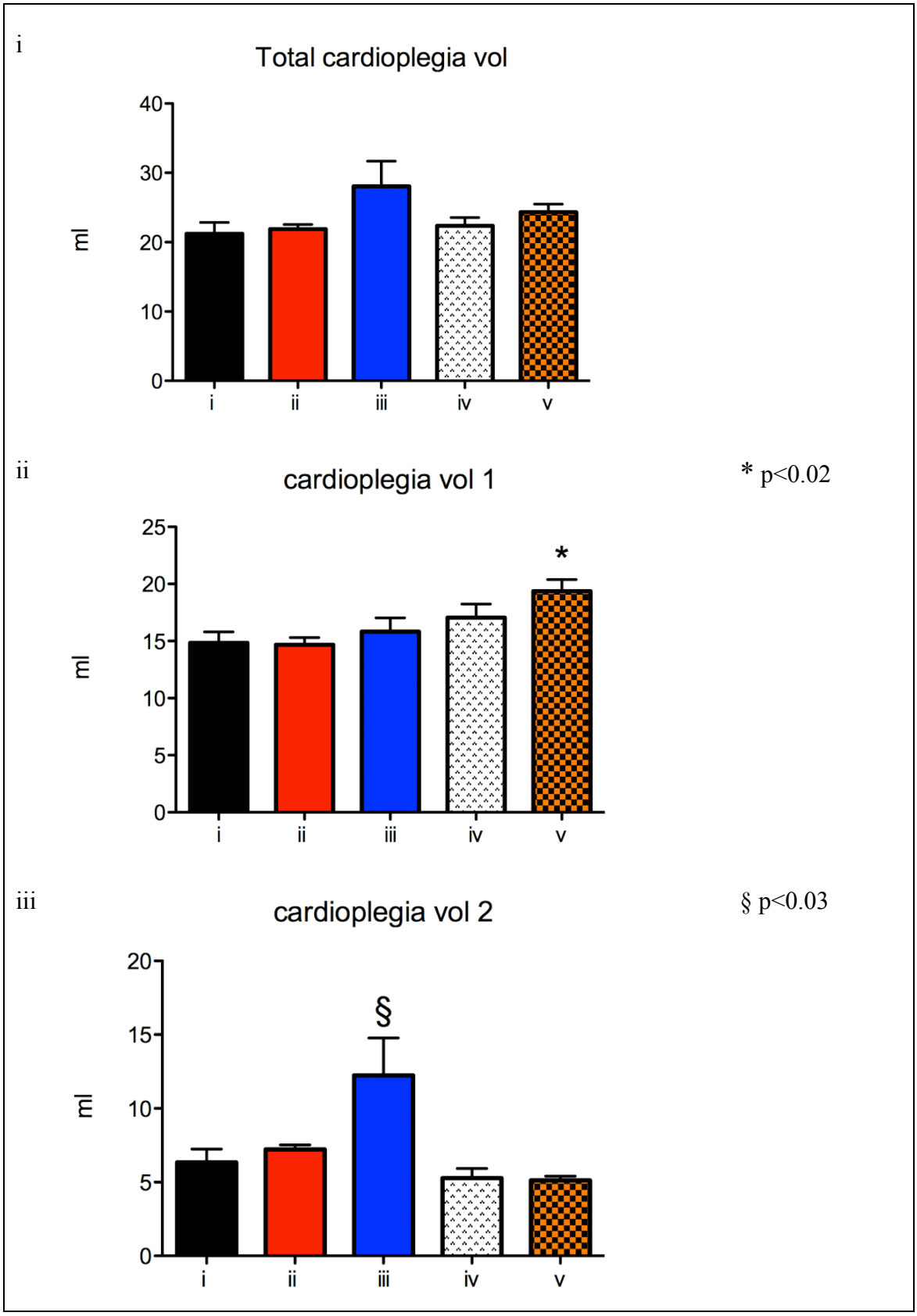


Figure 7.7 Volume of cardioplegia infused (i) total, (ii) first infusion at the start of ischaemia and (iii) second infusion during mid-ischaemia (n=6/group). * p<0.02, § p<0.03

7.4 Discussion

The evidence relating to the effects of cardioplegic protection on activity of MMPs is sparse, and especially the effect of MMP2 in hearts during global ischaemia-reperfusion and its impact on the recovery of the mechanical function of the LV. In human studies carried out by Spinale and co-workers [140], MMP activity was found to return to baseline levels with diastolic arrest of the heart with cardioplegia. However, it was difficult to ascertain whether this was due to a direct effect of the cardioplegic solution or to the fact that hypothermia was also used as a cardioprotective strategy. The low temperature will undoubtedly affect the activity of biological enzymes. In our studies, hypothermia was not used.

It was difficult to draw any substantial conclusion in our study regarding the effects of MMP inhibition and the timing of inhibition on metabolic activity in the hearts during ischaemia. During ischaemia, the time taken to develop contracture and to reach peak contracture were both delayed in Group (iii), although the delay to reach peak contractures was not significantly different in Group (iii) compared to the other groups. The data suggested that MMP inhibition should be present before, during and after ischaemia to affect the metabolic activity in the infarcted myocytes during the subsequent global ischaemia.

In an experimental study, Yeh and co-workers [144] postulated that upregulation of pro-inflammatory genes via the nuclear factor (NF)-κB also activates MMPs, causing myocardial dysfunction associated with cardiopulmonary bypass and global ischaemia induced during surgery. They pretreated rabbits with curcumin, a food derivative known to inhibit the activation of NF-κB pathway, 2 hours prior to institution of cardiopulmonary bypass with cold cardioplegia protection and global ischaemia of 60 min. At 4 hours of reperfusion, they found that inflammatory markers in the plasma were reduced, together with reduced troponin I and attenuation of myocardial MMP2 and MMP9 at the end of reperfusion. This correlated with improved recovery of myocardial contractility in the curcumin-treated groups.

In our model, we examined the early phase of reperfusion i.e the initial hour of reperfusion following 60 min of global normothermic ischaemia, with normothermic

infusion of cardioplegia solution. The MMP inhibitor was delivered directly to the myocardium with cardioplegia, hence eliminating the issue of bioavailability, as might occur in subcutaneous injections of drugs.

The above results showed that, in terms of the trend of recovery, there was a significant difference between the groups. The difference is especially marked during the early reperfusion period (within 30 min of reperfusion); with significantly better recovery of LVDP in the group where MMP inhibition was present before, during and after ischaemia (Group iii) compared to the group when MMP inhibition was only present during and after ischaemia (Group v). This implied that MMP2 has a contributory role during both ischaemia and reperfusion in causing mechanical dysfunction in the infarcted hearts. This could have an impact clinically if these observations are also seen in patients. A faster and better recovery during the early post-operative period could imply less inotropic support and intensive care management. Nonetheless, with further reperfusion period, the recovery of the hearts seemed to converge somewhat and the final LVDP recovery achieved in each group was not statistically significant. Due to the different protocol of MMP inhibitor tested, there were too many groups generated. Hence, this makes the statistical analysis less powerful and robust. It would have been ideal to only compare a few groups at a time. But, different timing of MMP inhibitor added may have implications in the recovery of the heart, so it was essential that these different protocols were tested.

It may also be that St Thomas' cardioplegia protects the heart, even the infarcted hearts, adequately that 60 min of global normothermic ischaemia was not sufficient to induce considerable injury to the hearts. Perhaps, a longer ischaemic time, such as 90 min, that would have induced more ischaemic injury and thus lower recovery of function may have generated conditions where any additional protective effects of MMP inhibition could have been disclosed. Alternatively, St Thomas' cardioplegia may actually have an effect on MMP2 activity. It may have been useful to sample the myocardium at mid-ischaemia and reperfusion to study the activity of MMP2 and metabolic state of the infarcted hearts. The other disadvantage of the model is due to a more general nature of MMP inhibition by 1,10-phenanthroline. Perhaps, a MMP2-selective inhibitor such as 444285, may result in better cardioprotection with selective MMP2 inhibition.

It was interesting to note that the volume of second infusion of cardioplegia was significantly better in Group (iii), which incidentally had the best recovery profile. MMP2 inhibition may better protect the endothelium of the coronary arteries, hence resulting in better overall recovery of mechanical function.

In conclusion, St Thomas' cardioplegia solution protected the infarcted hearts sufficiently well, even with 60 min of global normothermic ischaemia, such that there was reasonable recovery of mechanical function with reperfusion. Addition of a MMP inhibitor, 1,10-phenanthroline, before, during and after ischaemia, resulted in a trend towards an improved recovery profile, although the final recovery did not achieve statistical significance. On the other hand, if MMP inhibition was only introduced during and after ischaemia, it seemed to have a detrimental effect on myocardial function. This suggests that MMP2 may be an active player in the myocardium during ischaemia as its inhibition prior to ischaemia seems essential to offer some degree of cardioprotection. More work in the future, involving longer ischaemic time such as 90 or 120 min of global ischaemia and by using irreversible and specific MMP2 inhibitor will need to be carried out to further define the effect of cardioprotection of hearts using St Thomas' cardioplegia with MMP2 inhibition.

8 DISCUSSION AND CONCLUSION

8.1 Thesis aims and objectives

Since the seminal discovery of MMPs by Gross and Lapiere [9], there has been rapid progress in MMP research. The role of MMPs, which were originally seen as destructive enzymes involved in the regulation of the ECM, has now evolved into essential cell-signalling regulators in acute pathophysiology, such as acute ischaemia-reperfusion injury in the heart. The effects of MMPs have been investigated in various settings of acute ischaemia-reperfusion as described in Chapter 1 [35, 42, 140, 142]. The present study was undertaken to investigate the influence of MMP2 in regional infarction followed by global ischaemia-reperfusion as would occur in patients with ACS undergoing urgent cardiac surgery. This group of patients is becoming increasingly more common in clinical practice as diagnostic tests become more sensitive and specific as well as better understanding of the pathophysiology leading to expeditious treatment. In the UK, patients who underwent CABG within a month of their MI had almost doubled over a 4-year period (between 2004 to 2008) [70]. Current ESC/EACTS guidelines encourage aggressive management of revascularisation, including surgical revascularisation in high-risk patients [67]. Yet, subjecting infarcted hearts to further global ischaemia during surgery may have its detrimental effects. This, in some ways, is manifested in the higher mortality seen in this group of patients. Molecular pathways such as MMPs, activated by the initial infarction, may render the myocardium more vulnerable to further ischaemic insult, especially if the insult is imposed shortly after the initial injury [42]. The duration of MI and cardiac surgery is suggested to influence surgical outcome as discussed in Chapters 1 and 5, but the threshold is contentious. Hence, as well as investigating the role of MMP2 in this setting, the influence of interval between an initial MI and global ischaemia was also explored.

8.2 The rat in vivo LAD ligation model and isolated Langendorff (heart) perfusion

Rodents were used in these studies for several reasons. It is cheaper and logistically feasible to carry out larger number of experiments. In vivo myocardial infarction model in rats by surgical LAD ligation is an established and reliable method to create consistent MI as mentioned in Chapter 3. The results presented in Chapter 3 confirmed that the final infarct size obtained in the hearts after LAD ligation was consistent.

Although it is common to occlude various coronary arteries during isolated Langendorff heart perfusion, as far as we are aware, there was no previous study with established infarcted hearts (>24 hours) perfused in the isolated Langendorff perfusion model. The stability studies carried out in Chapter 3 showed that infarcted hearts (7 days post LAD ligation) were stable over a 120 min aerobic perfusion period; comparable to perfusion of normal hearts. A slightly higher LVEDP was noted in infarcted hearts compared to normal hearts, but neither the LVEDP after 20 min of equilibration with aerobic perfusion nor the change in LVEDP at the end of the perfusion period was significantly different. Pressure-volume relationship in the hearts may be an over-simplified way of assessing the mechanical function and contractility of the myocardium, especially in infarcted hearts. There may have been asymmetrical changes in the LV geometry and stiffness [100]; in addition, presence of an inflated balloon may also alter the intrinsic contractility of the heart. Other more sophisticated way of assessing functional recovery such as length-tension relationship could be used, but previous work undertaken in the laboratory showed that it did not add much more information to the study [145]. Hence, we choose to accept and acknowledge the limitations of the technique. As all groups were subjected to the same treatment, it is still a valid way of comparison.

Although rodents may be suitable to establish a proof-of-principle, the results obtained here may not be directly extrapolated to human. The remodelling process following MI in rat hearts may be different to human; it may be shorter and hence in 7 days, there may be some degree of adverse remodelling in the LV, which could explain the results seen in Chapter 4, Section 4.3.2. Hence, the infarcted rat hearts used in this study may not accurately represent adult human infarcted hearts 7 days after MI.

8.3 MMP2 and acute mechanical dysfunction in infarcted hearts subjected to another global ischaemic burden

With the present study, we found that when systemic factors were removed from the study design, infarcted hearts, when subjected to global ischaemia-reperfusion 7 days after the initial MI, had less capacity to recover its contractile function in an isolated heart model, compared to infarcted hearts subjected to the same ischaemic insult 3 days after the initial MI. As highlighted in the discussions in Chapter 4, Sections 4.1 and 4.4, different clinical series reported different outcomes regarding the mortality of CABG and the interval between MI and surgery [66, 71, 101, 103-106]. It is very difficult to accurately dissect out the important factors causing the increased mortality. Patient groups were very heterogenous and the clinical presentation and haemodynamic status at presentation and prior to surgery were also very diverse. Hence, our model offers the advantage of removing all these confounding factors, with focus solely on the biology of the infarcted myocardium as a variable. The findings were unexpected. One would have thought that with longer interval between MI and global ischaemia, the infarcted hearts would have had more time to recover and will be able to withstand the subsequent insult better. The second window of protection may explain the result we see in better recovery of function in infarcted hearts, when subjected to global ischaemia 3 days after MI [146]. The ischaemic triggers initiate a cascade of signalling of events resulting in protein synthesis, post-translational protein modification and a change in compartmentalisation of existing proteins, which in the second window of protection lasts for up to 72 hours following the ischaemic insult.

When we correlated the physiological effects with molecular events, specifically with myocardial MMP2 activity, we did find that MMP2 activity was significantly elevated in the infarcted hearts (Chapter 5). Petersen and co-worker showed that MMP2 protein level was elevated at day 1 post MI and remained elevated for a few weeks [33], but it is not known what happens to the protein level or the activity of MMP2 in the first week following MI. Unfortunately, due to the constraint of time, MMP2 activity was only interrogated in infarcted hearts 7 days after the initial MI. It would have been very informative to investigate MMP2 activity in infarcted hearts 3 days after MI to see how it correlates with the physiological phenomenon.

8.4 Assay of MMP2 activity

There are numerous ways to assay MMP2 and its activity [86, 120]; the most appropriate assay chosen depending on the objectives of the study. As this study primarily explores the role of MMP2 in mechanical dysfunction in infarcted hearts subjected to further global ischaemia-reperfusion, assays using native proteins, such as substrate zymography or immunocapture assays, are most suitable. Zymography was not used as the principal assay as the detection of gelatinolytic activity was based solely on molecular weight. Hence, an immunocapture assay was used in the study as it captured active MMP2, regardless of the molecular weights.

The other significant problem with either assay was that both are in-vitro assays. Therefore, it does not reflect the true activity of MMP2 in the tissues as MMP2 complexed to its endogenous inhibitors such as TIMPs, may be dissociated during tissue processing and during the assay itself. The assays carried out this way with whole heart tissue homogenisation does not give us any information about the location of active MMP2 within the myocardium. MMP2 may be activated at different cellular location such as the mitochondria or in the nucleus, and at different times during ischaemia and reperfusion. It may be better to use in-situ assay such as microdialysis method using fluorogenic substrates. It may provide more information about the overall MMP2 activity level in the tissues and the localisation of MMP2 activity. Unfortunately, these techniques are more complicated and costly in terms of equipment, and it was not readily available in the laboratory currently. Nevertheless, the in-vitro assays used were adequate to achieve the aims set out in this study.

8.5 Cardioplegia studies in infarcted hearts with and without MMP inhibitor

There were not many studies investigating the effects of cardioplegia on MMP activity [139, 140, 142]. Our data suggested that MMP2 has a role to play in causing mechanical dysfunction in infarcted hearts when subjected to further global ischaemia-reperfusion. Hence, the possibility that MMP inhibition could be used in conjunction with cardioplegia solution to protect the ischaemic heart better to withstand further ischaemic

insult was an exciting prospect. Different protocols of MMP inhibition were designed as the timing of MMP inhibition may impact the functional recovery of the infarcted heart [123]. We found that MMP2 inhibition must be present before, during and after ischaemia to confer better cardioprotection of the heart, especially during the early phase of reperfusion; allowing a more rapid recovery rate of the mechanical function of the heart compared to hearts where MMP2 inhibition was only present during and after ischaemia.

Given these results, it may have been prudent to further investigate the effect of cardioplegia on MMP2 activity. It would have been useful to assess myocardial MMP2 activity at various times of the perfusion protocol with and without cardioplegia protection; such as after aerobic equilibration, at mid-ischaemia and at early reperfusion.

8.6 Limitations

We acknowledge that there are inherent limitations with the experimental set-up and protocols and most of these were discussed in the relevant chapters. We have tried to design a suitable animal model, using the most suitable resources available to us, to study a clinically relevant issue. This, we hope will allow better integration of pre-clinical and clinical research so that we can maximise the output of research, as advocated recently by various researchers [59, 89, 147].

One essential disadvantage of the study is the use of small animal, rodents, in this case. There are bound to be subtle differences in certain molecular pathways and metabolic activities compared to the physiology and the responses to pathological processes in the human. The rats we used were young adult, male rats. These rats were not equivalent to a typical 65 year-old man who presents with ACS, requiring surgery within 7 days of their ACS. The fundamental pathological changes and processes within the myocardium and endothelium will be absent in the rodent species, with the absence of co-morbidity such as hyperlipidaemia, diabetes and hypertension. We will also not know the influence and interaction of various cardiovascular medications such as statins and anti-hypertensive drugs on the MMP2 activity in this context.

Besides, although isolated Langendorff heart perfusion confers the benefits of removing the confounding effect of systemic interaction, it is also a distinct disadvantage as these interactions may be important and may have significant impact on the recovery of the heart following acute ischaemia-reperfusion.

8.7 Future work

There are a lot more questions that need to be answered. A more thorough investigation of the haemodynamic status of the infarcted hearts could be carried out using transthoracic echocardiography and invasive pressure-volume catheterisation. This would allow us to document the serial haemodynamic and regional work changes in the LV from MI to just prior to excision for ex-vivo heart perfusion. Further characterisation of MMP2 activity in early infarct (i.e 3 days post LAD ligation) hearts during subsequent global ischaemia-reperfusion needs to be carried out. This way, we will be able to see how MMP2 activity in infarcted heart changes within the first 7 days of LAD ligation and whether, the changes in the activity at day 3 and day 7 post ligation may explain the mechanical dysfunction seen in Chapter 4, Section 4.3.2. It may also be more informative to have a way of assessing the in-situ MMP2 activity within the myocytes. Additionally, the activity during ischaemia itself may clarify further the role of MMP2 during the ischaemic period.

The effect of irreversible and specific MMP2 inhibition should also be explored as an adjunct to St Thomas' cardioplegia in cardioprotection of infarcted hearts. The ischaemic time should be extended to either 90 or 120 min and the infarcted hearts should also be sampled at baseline aerobic perfusion, during mid-ischaemia, immediately after ischaemia and at 10 min of reperfusion to assess MMP2 activity in the heart with St Thomas' cardioplegia with and without MMP2 inhibition. This may show us the effect of St Thomas cardioplegia on MMP2 activity in the infarcted hearts and whether MMP2 inhibition will make any significant impact.

8.8 Conclusion

In conclusion, in these studies, we attempted to examine a relevant and significant clinical problem using a simple experimental technique based entirely in the laboratory.

We have established that it is feasible to use acute in-vivo surgical LAD ligation model to induce MI in rat hearts and subsequently harvest the hearts within 7 days of MI for use in ex-vivo heart perfusion. The preparation is stable over at least a 120-min perfusion period, without significant deterioration in function. This is an invaluable model that allows us to study in detail biological and molecular events within the hearts, and relating these events to physiological function, when subjected to 2 separate but subsequent ischaemic stress. We conclude that infarcted hearts, when subjected to further global ischaemia-reperfusion injury, had less capacity to recover its contractile function, and that the impact was more significant in infarcted hearts 7 days after LAD ligation compared to 3-days post ligation. The functional impairment was associated with higher myocardial MMP2 activity. Inhibition of MMP2 ameliorated the dysfunction leading to improved recovery of mechanical function. Within the limitations of the experimental design, we see an inference in the link between the physiological effects and molecular events, further strengthened by functional improvement with molecular evidence of MMP2 inhibition.

MMP inhibition, when present before, during and after ischaemia, in hearts protected by St Thomas' cardioplegia, also resulted in significantly faster and better recovery during the early reperfusion period compared to hearts when MMP inhibition was only present during and after ischaemia.

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